Ana Sofia Rosa Santos Alves

USE OF BENTHIC MEIOFAUNA IN EVALUATING MARINE ECOSYSTEMS' HEALTH: HOW USEFUL CAN FREE-LIVING MARINE NEMATODES BE FOR ECOLOGICAL QUALITY STATUS (EQS) ASSESSMENT IN TRANSITIONAL WATERS?

Tese de Doutoramento em Biocências, ramo de especialização em Ecologia Marinha, orientada pelo Professor Doutor João Carlos Marques e co-orientada pela Professora Doutora Maria José Costa e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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Use of benthic meiofauna in evaluating marine ecosystems' health: How useful can free-living marine nematodes be for Ecological Quality Status (EQS) assessment in transitional waters?

Doctoral thesis in Biosciences, specialty in Marine Ecology, supervised by Professor Doutor João Carlos Marques and co-supervised by Professora Doutora Maria José Costa, presented to the Department of Life Sciences of the Faculty of Sciences and Technology of the University of Coimbra.

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CONTENTS

ABSTRACT

Meiobenthos is an important component of estuarine systems since it facilitates biomineralization of organic matter, enhances nutrient regeneration, serves as food for higher trophic levels and exhibits high sensitivity to environmental changes. Recently, the role of meiobenthos and nematodes as indicators of ecological quality and their integration in impact and monitoring studies has been valued, being essential to understand the distribution patterns of these communities.

In the scope of the growing awareness of the threat human activities represent to aquatic ecosystems, there has been a development in environmental policies, mainly focused on the ecological quality assessment. Research developed in this thesis had as main objective to enhance the knowledge regarding the ecological status and functioning of estuarine systems, based on the analysis of meiobenthic and free living nematode communities, both from subtidal and intertidal habitats. The Mondego estuary (Portugal) was addressed as case study.

In Chapter 1 the analysis of the ecological assessment information regarding macrofauna and nematode communities was performed in order to discern if these communities could provide a similar classification of the system. Along the estuarine gradient both macrofauna and meiofauna communities were simultaneously analyzed. The ecological status of the system was determined by the application of specific indices, with the results pointing towards a different trend regarding the analyzed communities. This comparative study showed that nematode and macrofauna provide different but complementary responses regarding environmental status, which may be explained by different response-to-stress times of each benthic community. Both assemblages should be integrated in monitoring studies to grant a more accurate assessment.

In Chapter 2 the analysis was focused on the spatial and temporal distribution of meiobenthos and nematode communities, aiming at determining the main structuring factors of their distribution. It was possible to validate the division of the estuarine gradient in different stretches and to verify that, at the analyzed spatial scale (the whole estuary, thus encompassing the entire estuarine

gradient), the effects of temporal variability were not translated in community variations, indicating that natural variability is also superimposed to the anthropogenic pressures present in some areas of the estuary.

Building on the results and interpretation of the work presented in Chapter 2, a thorough analysis of the taxonomic and functional structure of the subtidal nematode communities was carried out in Chapter 3, aiming at disentangling how the taxonomic and functional characteristic vary spatially and temporally and if there would be an added benefit in combining these approaches. This study allowed for a characterization of the traits structure of nematodes to be done for the first time for the Mondego estuary. It also allowed refining the interpretation of the estuarine stretches division, emphasizing that the upstream areas present a different community composition, something that is paramount when applying management tools. Additionally, although the Biological Traits Analysis was no more powerful than the traditional taxonomic approach in detecting spatial differences, it highlighted the peculiarity of some areas in terms of their functional structure increasing the knowledge and characterization of nematode communities in the estuary.

Finally, in Chapter 4, following an eutrophication mitigation measure applied in the South arm of the Mondego estuary, the response of intertidal meiofauna and nematode communities was assessed. At this small spatial scale (polyhaline stretch), the seasonal effects were superimposed to the spatial ones, not allowing discerning communities from areas where eutrophication symptoms are known to be different. Furthermore, it allowed the recognition of the impact of climatic events over meiobenthic communities.

A general discussion is also presented, integrating a synthesis of the thesis contributions to the knowledge on the use of meiobenthos and particularly free living nematodes to assess the ecological status and functioning of estuarine systems, and suggesting future research questions, challenges and paths.

Keywords: Estuary, estuarine gradient, meiobenthos, free living nematodes, ecological quality assessment, ecological indicators.

RESUMO

As comunidades de meiofauna e nemátodes têm um papel muito importante nos ecossistemas, estando envolvidas em processos de biomineralização de matéria orgânica, de regeneração de nutrientes, servindo de alimento para níveis tróficos superiores e exibindo uma elevada sensibilidade a perturbações ambientais. Recentemente o seu papel como indicador de qualidade ecológica e a sua integração em estudos de monitorização e impacto ambiental têm sido valorizados, sendo por isso essencial conhecer os padrões de distribuição das comunidades.

No contexto da crescente consciência da ameaça que as atividades humanas representam para os ecossistemas aquáticos, tem havido uma evolução nas políticas ambientais para se focarem principalmente na avaliação de qualidade ecológica. O trabalho de investigação desenvolvido nesta tese teve como principal objetivo aumentar o conhecimento do estado ecológico e funcionamento de sistemas estuarinos com base na análise das comunidades de meiofauna e nemátodes de vida livre, tanto em habitats subtidais como intertidais. O estuário do Mondego (Portugal) foi usado como caso de estudo.

No Capítulo 1 avaliou-se se as comunidades de macrofauna e nemátodes fornecem informação ecológica semelhante sobre o sistema. Ao longo do estuário do Mondego analisou-se, em simultâneo, comunidades de macroinvertebrados e meiofauna, com especial ênfase em nemátodes. Aplicando índices desenvolvidos para cada comunidade que visam analisar o estado ecológico do sistema, verificou-se que a informação fornecida pelas comunidades não seguia a mesma tendência. De facto, este estudo comparativo mostrou que macrofauna e meiofauna podem fornecer informação diferente mas complementar, uma vez que apresentam também diferentes tempos de resposta a perturbações, sendo aconselhado o seu uso complementar em estudos de monitorização.

O Capítulo 2 focou-se na análise da distribuição espacial e temporal de meiofauna e nemátodes ao longo do estuário do Mondego, com o objetivo de identificar os principais fatores ambientais relacionados com a sua distribuição. Verificou-se que o gradiente estuarino foi seguido pelas comunidades, não se

verificando, à escala espacial da análise, um efeito da variabilidade temporal sobre as mesmas. Este estudo evidenciou também o efeito da variabilidade natural sobre as pressões antropogénicas presentes no estuário.

Com base nos resultados do Capítulo 2, foi feita uma análise das características taxonómicas e funcionais das comunidades de nemátodes no Capítulo 3, aprofundando o seu conhecimento e analisando a sua distribuição espacial e temporal. Com este estudo foi feita uma análise das características ("traits") de nematódes pela primeira vez para o estuário do Mondego. Foi possível aprimorar a interpretação da divisão em diferentes áreas do estuário, com especial destaque para as áreas a montante, sendo esta informação útil quando se aplicam ferramentas de gestão. Além disso, embora a análise de características biológicas não tenha sido mais poderosa do que a abordagem taxonómica na deteção de diferenças espaciais, evidenciou a peculiaridade de algumas áreas em termos da sua estrutura funcional, aumentando o conhecimento e caracterização das comunidades de nematódes no estuário.

Por fim, no Capítulo 4, analisou-se a resposta das comunidades intertidais de meiofauna e nemátodes após a aplicação de uma medida de mitigação no Braço Sul do estuário do Mondego. À pequena escala espacial da análise (área polihalina) os efeitos da sazonalidade foram sentidos, com variações na comunidade, não permitindo distinguir claramente as comunidades de nemátodes ao longo do gradiente de eutrofização. Foi também possível confirmar o impacto de eventos climáticos na estrutura das comunidades.

A secção final de discussão geral integra e discute o uso das comunidades meiobentónicas para a avaliação do estado ecológico e funcionamento de sistemas estuarinos. Na sequência dos estudos feitos são também sugeridas novas abordagens e futuros desafios com vista a aumentar o conhecimento científico sobre estas comunidades e sua aplicação.

Palavras-chave: Estuário, gradiente estuarino, meiofauna, nemátodes de vida livre, avaliação de estado ecológico, indicadores ecológicos.

General introduction

GENERAL INTRODUCTION

> "As marine scientists we need to increase our own emphasis and pressures on behalf of the majority of species which do not have any appeal whatsoever, which are not attractive and which, for the most part are not even seen, yet which are the crucial elements of our biosphere."
>
> Sheppard, 2006

1. Estuaries: natural challenges for estuarine communities

Estuaries, as transition zones between freshwater and marine systems, are naturally variable ecosystems. The high degree of variability in the physical-chemical characteristics, such as salinity, dissolved oxygen, temperature and others, makes estuaries more variable than coastal and marine areas. In addition, the combination with variable bed sediment characteristics constitutes a great biological challenge to organisms inhabiting estuaries (Elliott and Quintino, 2007). Even so, it is widely accepted that estuaries are among the most productive and valuable natural systems around the world (Costanza et al., 1997; Jørgensen, 2010). Due to the influence of both sea and freshwater, estuaries are typically composed by different habitat types, which are physically, chemically and biologically interlinked (Meire et al., 2005), and may combine habitats like salt-marshes, seagrass beds, hard, and soft bottoms. These characteristics allow estuarine systems to provide essential breeding, nursing, and shelter grounds for invertebrates, fish and birds (e.g. Boström and Bonsdorff, 1997; Heck et al., 2003; Mander et al., 2007), as well as essential goods and services for humankind, which include water supply, climate regulation, nutrient cycling, erosion control, recreational and cultural uses (Costanza et al., 1997).

Owing to their resources and economic importance, estuaries are also among the most heavily modified habitats in the world (Lotze et al., 2006), with human activities being responsible for, amid other impacts, habitat loss/alteration, changes in the structure and functioning of biological communities and degraded

water quality (Kennish, 2000; McLusky and Elliott, 2004; Worm et al., 2006). Furthermore, eutrophication has become a wide-spread phenomenon, mostly linked to high nutrient influxes, as a result of several anthropogenic activities (Paerl, 2006), causing changes and negative effects of the biota.

Being naturally stressed areas and continuously subjected to high degrees of anthropogenic stress, estuaries present biological communities that have to cope with these pressures. According to Elliott and Quintino (2007) there is a similarity regarding organisms and assemblages from estuarine naturally stressed (where environmental factors change across the estuarine gradient) and anthropogenically stressed areas, making difficult to distinguish natural from human-induced stress in estuaries – this is what is termed as "Estuarine Quality Paradox" (Elliott and Quintino, 2007). The "Estuarine Quality Paradox" has repercussions for the implementation of environmental management plans, which rely on the definition of reference conditions (Elliott and Quintino, 2007), and is of particular relevance when using ecological indicators to determine the Ecological Status of transitional waters. In order to overcome this, several authors have suggested the use of specific methods, covering the entire biological system, especially its functioning and species composition (Hooper et al., 2005; de Jonge et al., 2006). In fact, several studies have also demonstrated the fundamental advantage of a multi-species approach, with the inclusion of many taxonomic and functional groups that have a broad range of sensitivities to any given environmental regime (Attrill and Depledge, 1997).

2. Assessing and managing natural and anthropogenic induced changes

Increasing pressures on aquatic ecosystems have been reported worldwide as a result of multiple stressors both from natural and anthropogenic origins (Dauvin, 2007). In fact, societal development increases pressures on ecosystems, challenging scientists to harmonize development and environment conservation. There has never been a greater need for scientific advice for management of aquatic systems (Schratzberger, 2012). The awareness of the threat that human activities represent to aquatic ecosystems led to the development and

implementation of more ambitious environmental policies in order to protect, conserve and manage the environment (Borja et al., 2008), moving towards an integrative management concept. Furthermore, several studies highlight the necessity for an improved understanding of the functioning of the systems and for new scientific knowledge to inform, in a more effective way, decision-makers and the public (e.g. Lubchenco, 1998; Hooper et al., 2005; Schratzberger, 2012).

In Europe, the Water Framework Directive (WFD; 2000/60/EC) and the Marine Strategy Framework Directive (MSFD; 2008/56/EC), relate the assessment of ecological quality within marine (i.e. estuarine and coastal waters) and offshore waters, respectively, ensuring that human activities are carried out in a sustainable way (Borja et al., 2008). Actually, the WFD introduced a new concept of water management in the European Union. Aiming at achieving the "Good Ecological Status" for all water (surface and groundwater including transitional and coastal waters) by 2015, this Directive establishes an outline for the protection and improvement of all European waters. The concept of environmental status takes into account the structure, function and processes of the systems, bringing together natural physical, chemical, physiographic, geographic and climatic factors, integrating these conditions with the anthropogenic impacts and activities in the concerned area (Borja et al., 2008). Hence, the concept of ecological quality is defined in an integrative way, by using several biological parameters, together with physicochemical and pollution elements (Borja et al., 2008). These integrative tools are meant not only to assess the ecosystem quality but also to provide communities and decision-makers with tools to define and monitor the evolution, current condition and biological performance of ecosystems (Borja et al., 2008). In fact, sampling of physicochemical or abiotic variables to detect a change or impact may be problematic (Goodsell et al., 2009) and concentrations of contaminants may be too small to be detected (Suter, 2001), being recognized the advantage of using biological rather than physicochemical indicators (Goodsell et al., 2009) to measure environmental pollution and impacts. Due to the integration of both biotic and abiotic components of an ecosystem through their adaptive responses, living organisms are the most appropriate indicators for use in the evaluation of a system (Casazza et al., 2002).

3. Meiobenthic research: trends and challenges

Environmental assessment uses a fauna group that is considered appropriate, either because we value it in some way or it has intrinsic value (as performing essential ecosystem functions), or because it is a good indicator of environmental changes (Schratzberger, 2012). Community-based approaches, especially those involving macrobenthic invertebrates, have always been favoured as indicators of aquatic assessments over meiofauna (Schratzberger et al., 2000), mainly because taxonomic keys and sampling protocols for the former are well documented (Schratzberger, 2012), and due to the organisms well-known features and their fairly quick responses to both natural and anthropogenic stress (Pearson and Rosenberg, 1978; Dauer et al., 2000; McLusky and Elliott, 2004).

Nonetheless, as a result of their close association with the substrate, high diversity and importance in ecosystem functioning, meiofauna and free-living nematodes are useful indicators in a variety of cases, with recent studies addressing key ecological issues such as processes that underpin faunal distribution patterns and their importance in the trophic dynamics of aquatic ecosystems (Schratzberger, 2012). They are thus, extremely useful in assessing the effects of anthropogenic disturbance in aquatic sediments (Heip et al., 1988; Coull and Chandler, 1992; Kennedy and Jacoby, 1999, Schratzberger et al., 2000).

Due to its peculiar characteristics such as the ubiquitous occurrence, high abundance, high turnover of generations and fast metabolic rates, meiofauna communities can be advantageous, over most macrofauna, in reflecting the overall health of the systems (Giere, 2009). Actually, nematodes are able to maintain populations in extreme physical conditions where other taxa, especially macrofaunal taxa, are eliminated (Heip, 1980), allowing different degrees of disturbance to be detected even when macrofauna ceased to be present (Boucher and Lambshead, 1995). Nematodes play an important role in the structure and functioning of aquatic ecosystems (Heip et al., 1985) and due to their high structural and functional diversity, are appropriate to be used in biomonitoring studies as they are suitable indicators of pollution-induced disturbances of benthic

ecosystems (Coull and Palmer, 1984; Coull and Chandler, 1992; Bongers and Ferris, 1999; Höss et al., 2011; Moreno et al., 2011).

3.1. Meiobenthos and nematodes as bio-indicators

As a consequence of their common and widespread occurrence (even in areas where macrobenthos are scarce or inexistent), high abundances, high taxonomic diversity, benthic larvae and short life cycles, meiofauna can easily respond to environmental changes and disturbances resulting from both natural and anthropogenic events. Although their response to disturbance is highly variable among species and communities, nematode assemblages are most affected by the kinds of disturbance that they do not experience in naturally stressed environments (Schratzberger and Warwick, 1999a). Their changes in density, diversity, structure and functioning, when stressed, are ideal to "detect" changes in the systems (e.g. Soetaert et al., 1994; Li et al., 1997; Essink & Keidel, 1998; Schratzberger and Warwick, 1998a; Steyaert et al., 2003; Schratzberger et al., 2004). These "qualities" justify why the use of meiobenthos and nematodes in quality assessment studies has been highly recommended (e.g. Schratzberger et al., 2000; Moreno et al., 2011, Patrício et al., 2012; Alves et al., 2013) even though seldom used.

Actually, there are ecological and practical advantages associated with using nematodes in benthic biological studies (Schratzberger et al., 2000). Briefly, the small size of meiobenthic communities allows their maintenance in small volumes of sediment, allowing repeated sampling with minor disruption of sampling sites. Furthermore, it allows the follow-up of small-scale experiments using nematodes in the laboratory, under controlled and repeatable conditions. Their high abundance and diversity gives a significant intrinsic information value to each sample and ensures statistical validity of the data. The high diversity of nematode assemblages suggests a high degree of specificity in the choice of the environment, while their short generation times (most species present life-cycles of one to three months) makes changes in the community structure to be detected in short-terms studies. Furthermore, their direct development (and sessile life cycle) provides information on the effects of contaminants in the sediment as the animals are in

direct contact with solvents in the interstitial water through their permeable cuticle. Although the innumerous advantages, some limitations are also reported as *i*) taxonomic problems in the identification of individuals with small bodies, being necessary a high-power microscope for species identification, *ii*) community response of meiofauna to environmental perturbations are not well documented (inexistence of extensive literature to compare), *iii*) the high abundance and diversity, together with the lack of taxonomic expertise make the analysis of meiofauna community structure a time-consuming and labour-intensive task, *iv*) population density is affected by a variety of abiotic and biotic factors and due to its patchy distribution pattern, meiofauna density may fluctuate over distances of a few centimeters (Schratzberger et al., 2000).

According to Kennedy and Jacoby (1999) and Goodsell et al. (2009), nematodes are the ideal group to utilize in the assessment of sediment "quality", emphasizing the conclusions of Bongers and Ferris (1999), which state that if environmental scientists had to draft a group of organisms that would specifically serve to monitor and measure biodiversity and the impact of stressors, then the blueprint for those organisms would certainly closely match the characteristics of nematodes.

Therefore, although the general perception that "meiofauna are not impressively large or tasty and they are not even dangerous – they are simply small" (Giere, 2009), deems them uninteresting to most people, their productive capacity, ecological adaptability and environmental sensitivity is of great interest (Giere, 2009), especially to assess the structure and function of ecosystems. While not seen as primary target, meiofauna are a very valuable instrument to address key ecological issues (Schratzberger, 2012).

3.2. Meiobenthic communities: definition and composition

The term meiofauna was firstly introduced by Mare (1942) to define an assemblage of benthic metazoans of intermediate size that could be distinguished from "macrobenthos" by their small sizes, but were larger than the "microbenthos" (bacteria, diatoms and most protozoa). Used as a synonym of meiofauna, "meiobenthos" are defined, on a methodological basis, by the formal size

boundaries based on the standardized mesh width of sieves, with 1 mm (a 0.5 mm sieve may also be used) as upper limit and 44 μm (63 μm) as lower limit. However, these limits are not strict and, for instance, deep sea studies use smaller mesh sizes (31 μm) in order to retain even the smallest meiofauna organisms (Giere, 2009). Meiofauna represents thus a separate, biologically and ecologically, defined group of animals (Schwinghamer, 1981; Warwick, 1984), composed by organisms with a biomass size spectrum (dry adult body mass) ranging from 0.01 to 50μg and having a coherent set of life-history and feeding characteristics, setting them apart as a separate evolutionary unit (Warwick, 1984).

Meiofauna are a taxonomically and morphologically diverse group representing a wide range of invertebrate taxa. The dominant taxa are usually nematodes (Nematoda) and harpacticoid copepods (Crustacea Copepoda), with other important groups including turbellarians (Platyhelmintes Turbellaria), ostracods (Crustacea Ostracoda), gastrotrichs (Gastrotricha), tardigrades (Tardigrada), rotifers (Rotifera), polychaetes (Annelida Polychaeta), oligochaetes (Annelida Oligochaeta), mites (Arachnida Acarina), gastropods and bivalves (Mollusca Gastropoda and Bivalvia), and many others with lower presence (Urban-Malinga, 2013).

3.3. Nematode communities: biological and ecological characteristics

Free-living nematodes are the numerically dominant metazoan representatives of the benthos of many marine and brackish-water habitats, usually consisting of 80-95% of the individuals and 50-90% of the biomass (Higgins and Thiel, 1988; Giere, 2009). There are 4000-5000 known and described species of free-living marine nematodes worldwide (Eyualem-Abebe et al., 2008). However, the diversity of nematodes, assessed by number of species, is hampered by the fact that many species remain undiscovered and by the existence of criptic diversity in some taxa (e.g. *Terschellingia*, Bhadury et al., 2008). Thus, global estimates for the total number of species vary from 10000-20000 species (Mokievsky and Azovsky, 2002) up to more than 1 x 10^6 species (Lambshead, 1993; Snelgrove et al., 1997). Furthermore, the phylogenetic relationships of

nematodes (given by De Ley et al., 2006 and Meldal et al., 2007) are far from stable, being necessary more genetic, morphological and ultrastructural details to further resolve the natural phylogenetic units in nematodes. Genetic analysis for use in the systematics of lower nematode taxa can add valuable information in order to disentangle the diversity in highly specious nematode genera (mainly those which are problematic to assess based only on external morphology). The efforts being made to develop analysis of population genetics (Derycke et al., 2005) and DNA barcoding (Bhadury et al., 2006) aim at contributing to a more holistic approach by encompassing taxonomic, molecular and morphological approaches.

Nonetheless, the morphological approach is still being largely the first comprehensive step for the documentation of biodiversity (e.g. Derycke et al., 2008; Fonseca et al., 2008). Briefly, the identification process of nematodes is mainly based on characters that are visible at a compound microscope. The general body and tail forms, the buccal cavity differences, cuticle patterns and structures, number and arrangements of sensory setae (particularly around the head), and the position and shapes of anphids (paired anterior chemical sense organs) allows the high species richness to be broken down in large groups as a crucial step towards taxonomic ordination.

Since nematodes are the main element in meiobenthic communities, it is not surprising that the distribution patterns of meiofauna and nematodes are mainly structured by the same variables, as well as their role in ecosystems mostly relates the same functions.

3.4. Distribution patterns of meiobenthic and nematode communities

Regardless of the sediment, meiofauna are always present in high densities, typically in the range of 10^5 to 10^7 ind.m^{-2}. They occupy a diverse range of habitats from freshwater to marine areas and from high on the beach to the deepest depths of water bodies (Higgins and Thiel, 1988). They are mostly found in and on soft sediments (essentially in the interstitial space between sand grains or burrowed in finer sediments), displacing sediment particles and changing the sediment texture,

but also among epilithic plants and other hard substrates (e.g. animal tubes) (Giere, 2009; Urban-Malinga, 2013).

Several factors affect the distribution patterns of abundance and biomass of meiofauna and nematodes, both at the horizontal and vertical levels. Grain size is a key factor in shaping meiofauna distribution by determining spatial and structural conditions and indirectly determining the physical and chemical milieu of the sediment (Giere, 2009). Additionally, tidal exposure, depth, season, nutrients and pollutants are also known to influence meiofauna distribution, with the highest values being typically observed in intertidal muddy estuarine habitats (Higgins and Thiel, 1988). At the horizontal level, the referred factors, their interactions (with counteracting, additive or synergistic effects) and biotic factors (food supply, predation, competition and reproductive strategies) can have a considerable influence on structuring meiofauna communities. Furthermore, habitat heterogeneity, caused by physical variations, by the activity of meiofauna food sources or by the activity of macrofauna, also has a determinant role in the high variability of the meiofauna communities (Coull, 1988).

In detail, the horizontal distribution patterns of marine nematodes can be investigated from small to global scales, being regulated by the complex interactions between hydrodynamic regime and physical and chemical proprerties in soft bottoms (Snelgrove and Butman, 1994; Giere, 2009). At the small scale (mm-cm) nematodes show an aggregated distribution, with patches depending on complex interaction between biotic and abiotic factors (Li et al., 1997), making difficult to model the distribution and diversity patterns of nematodes (Merckx et al., 2009). Furthermore, disturbance and predation generated by the feeding activities of some organisms may reduce nematode densities (Schratzberger and Warwick, 1999a; Danovaro et al., 2007), as well as the distribution of microphytobenthos, affecting nematode small scale spatial distribution (Montagna et al., 1983). At the mesoscale (m-km), nematode distribution patterns have been linked to variations in the physicochemical properties of the sediment, with grain size being one of the main factors related to the structure of the assemblages matrix (e.g. Findlay, 1981; Soetaert et al., 1994; Tita et al., 1999; Steyaert et al. 2003, Alves et al., 2009; Adão et al., 2009). Likewise, salinity and tidal exposure are

also important factors, being visible, for instance, a change in communities along estuarine gradients (Heip et al., 1985). At the large (global) scale, generalizations are still problematic since species distributions have been poorly studied. Furthermore, the comparison among studies is hampered by the different methodologies used for sampling and identification (Soetaert et al., 1995).

The vertical zonation of meiofauna and nematodes is mainly controlled by oxygen concentration and depth of the redox discontinuity layer, a boundary between aerobic and anaerobic sediments. In fact, oxygen concentration decreases with depth, towards the redox potential discontinuity (RPD), above the anoxic sediment (Gray, 1981). The depth of this layer is controlled by sediment grain size, with coarser sediments being more oxygenated and with a deeper RPD, whereas in finer sediments nematodes can be restricted to the first cm (Coull, 1988). Besides that, tides and current, directly affecting oxygenation of the interstitial water, are structuring factors, followed by bioturbation promoted by macrofauna and meiofauna that cause modifications in the sediment matrix (Vanreusel et al., 1995). The interaction of physical and biological factors varies according to sediment type, causing different patterns to arise. In muddy sediments, the majority of fauna is found in the upper 2 cms of the sediment, while in sandier sediments, more oxygenated, meiofauna can be found deep in the sediment (Vincx, 1996). In fact, muddy sediments usually present approximately twice as many meiofauna in the top first cm as the first 10 cms of sandy sediments (Smith and Coull, 1987).

In reality, in estuaries, different meiofauna assemblages may occupy different habitats: assemblages in mud differ from those in sand and the ones in low salinity may differ from the ones in high salinity (Soetaert et al., 1995).

3.5. Role in ecosystems

Besides being affected by the surrounding abiotic and biotic environment, meiobenthos and nematodes significantly influence the interstitial processes, controlling the magnitude of resources, affecting sediment stability and playing an important role in the structure and functioning of ecosystems (Heip et al., 1985; Snelgrove et al., 1997; Gray and Elliott, 2009; Urban-Malinga, 2013).

Briefly, the various roles of meiobenthos on sediments can be summarized as follows: *i*) the physical activity of meiofauna and grazing on diatoms destabilize the sediment and the bioturbation resultant from these activities enhances geochemical fluxes (mostly fluxes of oxygen and nutrients vital for microbial decomposition); *ii*) mucus produced by some taxa stabilizes the sediment and promotes microbial growth; *iii*) microbial feeders stimulate microbial activity and decomposition; *iv*) meiofauna mechanically breaks down detrital particles, making them more accessible and amenable to bacterial colonization and susceptible to bacterial degradation; *v*) decaying meiofauna constitutes food for bacteria and due to their rapid turnover rates, nutrients are rapidly returned to the system; and *vi*) meiofauna serves as food for higher trophic levels (e.g. macrofauna, juveniles of fish species) and, by feeding on them, other organisms are affected, controlling the magnitude of resources and affecting the structure and function of the whole benthic system (Heip et al., 1985; Snelgrove et al., 1997; Gray and Elliott, 2009).

It is comprehended that this benthic component affects thus several essential ecological processes such as regeneration of nutrients, transfer of energy to higher levels in the food webs and bioturbation of sediments (Giere, 2009), being essential, in order to understand the structure and functioning of benthic ecosystems, to investigate nematode communities.

3.6. Functional characterization of nematodes

Nematodes research is mainly focused on diverse research topics, ranging from latitudinal patterns of biodiversity (e.g. Mokievsky and Azovsky, 2002; Gobin and Warwick, 2006) and ecological factors driving the structure of assemblages (e.g. Soetaert et al., 1995; Schratzberger et al., 1998a; 1998b; Steyaert et al., 1999; Hua et al., 2009) to links between taxonomic diversity and functional traits (e.g. Schratzberger et al., 2007; Liu et al., 2011). In fact, the importance of the link between nematode diversity and ecosystem function has been highlighted (Danovaro et al., 2008), being recognized that changes in biodiversity may modify ecosystem function (Hooper et al., 2005), with taxonomic analyses alone omitting key functional aspects (Frid et al., 2000; Bremner et al., 2003). Actually, when attempting to evaluate the effects of environmental change, the inclusion of

functional properties has been recommended (de Jonge et al., 2006). According to Chalcraft and Resetarits (2003), species in the same functional groups share morphological traits that are thought or known to represent an important ecological function. Regarding nematodes, some studies have devoted attention to the ecological meaning of this morphological diversity (Tita et al., 1999; Vanaverbeke et al., 2003; Schratzberger et al., 2007) which, according to Giere (2009), is perhaps the most informative system used to connect the diverse biological requirements of nematodes with the functional dynamics of the community.

In fact, Schratzberger et al. (2007) analyzed nematode community functions and combined a set of selected morphological features (body size and shape, buccal structure, tail shape) with known biological traits, relating functional composition with the environmental characterization, and suggested that single measures which are only based on phylogenetic classification do not capture all the important differences in nematodes attributes. Furthermore, it has been encouraged the use of both taxonomic and biological traits approaches to provide additional insights from those obtained from the traditional taxonomic analyses (Alves et al., 2014). Nevertheless, it is also recognized that further knowledge of the functional roles of nematode species will be the key to improve the sensitivity and interpretation of biological traits analyses of benthic communities (Schratzberger et al., 2007; Alves et al., 2014).

4. General aims and thesis outline

The main aim of this thesis was to understand the role of meiobenthic and free-living nematode communities in temperate estuarine systems and to evaluate their potential role as ecological quality indicators, expanding our knowledge on their distribution constraints, ecological, and functional characterization while identifying critical features that could be used in an accurate classification of transitional systems.

To pursue and achieve the main objective, a group of studies was undertaken to respond to the following specific objectives:

- To analyze if nematode and macrofauna assemblages provide similar ecological assessment information;
- To assess the spatial and temporal distribution of meiobenthos and, more specifically, free-living nematodes in estuarine systems;
- To investigate the use of taxonomic classification and functional traits of nematodes regarding the detection of the main factors related to communities distribution patterns;
- To assess the ability of intertidal meiofauna and nematode communities as indicators of system's recovery processes.

To accomplish these objectives, specific topics were addressed, which gave origin to the four chapters composing the core structure of the thesis.

At the end, an integrative discussion is presented, summarizing the most relevant findings of this thesis. Furthermore, during the course of the thesis, several new questions were raised and revealed new paths that can and should be explored. A brief discussion on the questions that were left unanswered or that were raised by our main findings is thus presented.

The thesis is based on the following scientific papers:

Chapter 1

Patrício, J., Adão, H., Neto, J.M., Alves, A.S., Traunspurger, W., Marques, J.C., 2012. Do nematode and macrofauna assemblages provide similar ecological assessment information? Ecological Indicators 14, 124–137.
doi: 10.1016/j.ecolind.2011.06.027

Chapter 2

Alves, A.S., Adão, H., Ferrero, T.J., Marques, J.C., Costa, M.J., Patrício, J., 2013. Benthic meiofauna as indicator of ecological changes in estuarine ecosystems: The use of nematodes in ecological quality assessment. Ecological Indicators 24, 462-475.
doi: 10.1016/j.ecolind.2012.07.013

Chapter 3

Alves, A.S., Veríssimo, H., Costa, M.J., Marques, J.C., 2014. Taxonomic resolution and Biological Traits Analysis (BTA) approaches in estuarine free-living nematodes. Estuarine, Coastal and Shelf Science 138, 69-78.
doi: 10.1016/j.ecss.2013.12.014

Chapter 4

Alves, A.S., Caetano, A., Costa, J.L., Costa, M.J., Marques, J.C., Estuarine intertidal meiofauna and nematode communities as indicator of ecosystem's recovery following mitigation measures *(Submitted to Ecological Indicators)*.

Chapter 1

Do nematode and macrofauna assemblages provide similar ecological assessment information?

Do nematode and macrofauna assemblages provide similar ecological assessment information?

ABSTRACT

Do nematode and macrofauna assemblages provide similar ecological assessment information? To answer this question, in the summer of 2006, subtidal soft-bottom assemblages were sampled and environmental parameters were measured at seven stations covering the entire salinity gradient of the Mondego estuary. Principal components analysis (PCA) was performed on the environmental parameters, thus establishing different estuarine stretches. The ecological status of each community was determined by applying the Maturity Index and the Index of Trophic Diversity to the nematode data and the Benthic Assessment Tool to the macrofaunal data. Overall, the results indicated that the answer to the initial question is not straightforward. The fact that nematode and macrofauna have provided different responses regarding environmental status may be partially explained by local differentiation in microhabitat conditions, given by distinct sampling locations within each estuarine stretch and by different response-to-stress times of each benthic community. Therefore, our study suggests that both assemblages should be used in marine pollution monitoring programs.

Keywords: nematodes, macrofauna, estuarine gradient, ecological assessment, Portugal.

INTRODUCTION

The introduction of biological features in the assessment of environmental quality is one of the innovations of recent monitoring programs, as required by the Water Framework Directive of the European Union (WFD, 2000/60/EC). Regarding communities of benthic invertebrates, those of macrofauna have been traditionally used to assess and evaluate ecological integrity. In fact, organisms comprising the benthic macrofauna are considered to be good indicators of coastal and estuarine ecological conditions for several reasons (see Pinto et al., 2009 for detailed references), including their taxonomic diversity and the abundance of many taxa, their wide range of physiological tolerance to stress and the variability of their feeding modes and life-history strategies. These traits allow the benthic macrofauna to respond to a wide range of environmental changes. Moreover, these organisms are relatively sedentary and thus cannot easily escape unfavorable conditions, which makes them reliable indicators of local pressure. In addition, some taxa are relatively long-lived and thus reflect the effects of environmental conditions integrated over longer periods of time. In terms of their study, benthic macrofauna are relatively easy to sample quantitatively and, compared to other smaller sediment-dwelling organisms, they have been fairly well studied scientifically, with taxonomic keys available for most groups.

Specific indicators that can be used to determine macrofaunal abundance, diversity, and the presence/absence of sensitive species were proposed and subsequently tested in assessments of the environmental quality of coastal and estuarine systems (e.g. Borja et al., 2004; Rosenberg et al., 2004; Bald et al., 2005; Simboura et al., 2005; Muxika et al., 2007; Teixeira et al., 2009). Nevertheless, it may well be the case that meiofauna can also suitably reflect the ecological conditions present in a particular system. In fact, meiofaunal communities, namely those of nematode, have generated considerable interest as potential indicators of anthropogenic disturbances in aquatic ecosystems (e.g. Heip et al., 1988; Schratzberger et al., 2004; Gheskiere et al., 2005; Gyedu-Ababio and Baird, 2006; Hoess et al., 2006; Steyaert et al., 2007; Moreno et al., 2008). For instance, Kennedy

and Jacoby (1999) maintained that meiofauna has several potential assessment advantages over macrofauna, such as small size, high abundance, ubiquitous distribution, rapid generation times, fast metabolic rates, and the absence of a planktonic phase, resulting in a shorter response time and higher sensitivity to certain types of disturbance. Moreover, due to their ecological characteristics, meiofaunal organisms can act as suitable indicators of changes in environmental conditions over small spatial scales (e.g. Soetaert et al., 1994; Li et al., 1997; Steyaert et al., 2003). According to Bongers and Ferris (1999), if environmental scientists had to draft a group of organisms that would specifically serve to monitor and measure biodiversity and the impact of stressors, then the blueprint for those organisms would certainly closely match the characteristics of nematodes. However, while there are many general indices of biological diversity, only a few specific but limited tools have been developed for nematodes. Among these are the Maturity Index (Bongers, 1990), which is based on the allocation of taxa according to life strategy, ranging from colonizers (r-strategists in the broad sense) to persisters (K-strategists), and the Index of Trophic Diversity (Heip et al., 1985). Both have been widely used in environmental assessments based on nematode assemblages (e.g. Heip et al., 1985; Bongers et al., 1991; Soetaert et al., 1995; Gyedu-Ababio et al., 1999; Beier and Traunspurger, 2001; Danovaro and Gambi, 2002; Gyedu-Ababio and Baird, 2006; Moreno et al., 2008).

What if, in an alternative approach, the best characteristics of meiofauna and macrofauna could be taken advantage of to obtain complementary information allowing more precise environmental monitoring? Several studies have compared the response of meio- and macrobenthos community structure to disturbances and pollution (e.g. Warwick, 1988a; Austen et al., 1989; Warwick et al., 1990; Schratzberger et al., 2003; Austen and Widdicombe, 2006; Bolam et al., 2006; Whomersley et al., 2009; Widdicombe et al., 2009). As far as we know, in the few field studies in which the spatial patterns of meiofauna (or nematode) and macrofauna have been simultaneously compared, changes in both assemblages as a response to natural gradients were found to be scattered across a small number of habitats: a high-energy surf zone (McLachlan et al., 1984), glacial fjords (Bick and Arlt, 2005; Somerfield et al., 2006), a Brazilian atoll (Netto et al., 1999),

Brazilian mangroves (Netto and Gallucci, 2003), an abyssal site in the NE Atlantic (Galéron et al., 2001), NE Atlantic slopes (Flach et al., 2002), offshore of the West UK coast (Schratzberger et al., 2004; 2008), the Thames estuary (UK) (Attrill, 2002), Mediterranean sandy beaches (Covazzi et al., 2006; Papageorgiou et al., 2007), and the Eurasian Arctic Ocean (Kröncke et al., 2000). These investigations have demonstrated the fundamental advantage of a multi-species approach, with the inclusion of many taxonomic and functional groups that have a broad range of sensitivities to any given environmental regime (Attrill and Depledge, 1997). This is particularly true for estuarine systems, where assessment of the environmental ecological conditions must account for their greater natural variability. Transitional waters are indeed more complex than other categories of surface waters. Indeed, conditions in areas close to the mouth of the estuary, where the marine influence is strong, are highly distinct from the polyhaline and mesohaline inner parts of the estuary, and differ, in turn, from the oligohaline conditions and fresh tide influence found at the estuarine head (Elliott and McLusky, 2002). The natural stressors resulting from the presence of gradients such as these throughout the system could mask the response of potential indicators (Dauvin, 2007; Elliott and Quintino, 2007). Therefore, prior to the use of environmental quality assessment tools, the different components that make up the system should be accounted for.

The principal aim of this work was to determine whether subtidal nematode and macrofauna assemblages could provide a comparable assessment of ecological conditions. In addition, we examined whether both assemblages (with their own specific tools and approaches) were able to characterize a priori defined estuarine stretches, and compared the changes in nematode and macrofauna community structure that occurred along a natural estuarine gradient.

MATERIALS AND METHODS

Study site

The Mondego River basin comprises an area of approximately 6670 km^2, including a large alluvial plain consisting of high-quality agricultural land. The river's estuary (Fig. 1) (western coast of Portugal; 40º08'N, 8º50'W) is 21 km long and constitutes a relatively small (860 ha) warm-temperate polyhaline system. At a distance of 7 km from the sea, Murraceira Island splits the estuary into two arms with very different hydrological characteristics. The North arm is deeper (5–10 m during high tide) and is the river's main navigation channel, receiving most of the freshwater input (27 m^3 s^{-1} in dry years up to 140 m^3 s^{-1} in rainy years; mean annual average of 79 m^3 s^{-1}). It is therefore strongly influenced by seasonal fluctuations in river flow. The main pressures disturbing the Mondego's North arm mainly come from the facilities associated with the harbor at Figueira da Foz, specifically, dredging activities that cause physical disturbance of the bottom sediments. The South arm is shallower (2–4 m during high tide), with large areas of intertidal mudflats (almost 75% of the area) that are exposed during low tide (Neto et al., 2008). It is considered to be the richest area of the estuary in terms of productivity and biodiversity (Marques et al., 1993). According to Veríssimo et al. (2012a), the upstream areas (oligo and mesohaline stretches) are essentially characterized by higher nutrients concentrations, coming from the Mondego River's catchment area, especially direct runoff from the 15,000 ha of cultivated land (mainly rice fields) in the lower river valley (Neto et al., 2008; Teixeira et al., 2008). The South arm is mainly distinguished by fine sediments and higher sediment organic matter content and, in general, the downstream stretches show higher values of salinity, dissolved oxygen and transparency (Veríssimo et al., 2012a). Pereira et al. (2005) determined the concentration of major (Al, Si, Ca, Mg, Fe), minor (Mn), and trace elements (Zn, Pb, Cr, Cu, Ag, Cd, Hg) and organochlorine compounds in 24 stations along the entire estuarine area and concluded that all sediment samples showed low levels of contamination reflecting the weak industrialization of the region. Even though, the higher incorporation of elements was registered in muds deposit in the inner part of the South arm. In addition to

the aforementioned disturbances, the estuary also supports industrial activities, salt-extraction, aquaculture farms, and seasonal tourism activities that are centered around Figueira da Foz.

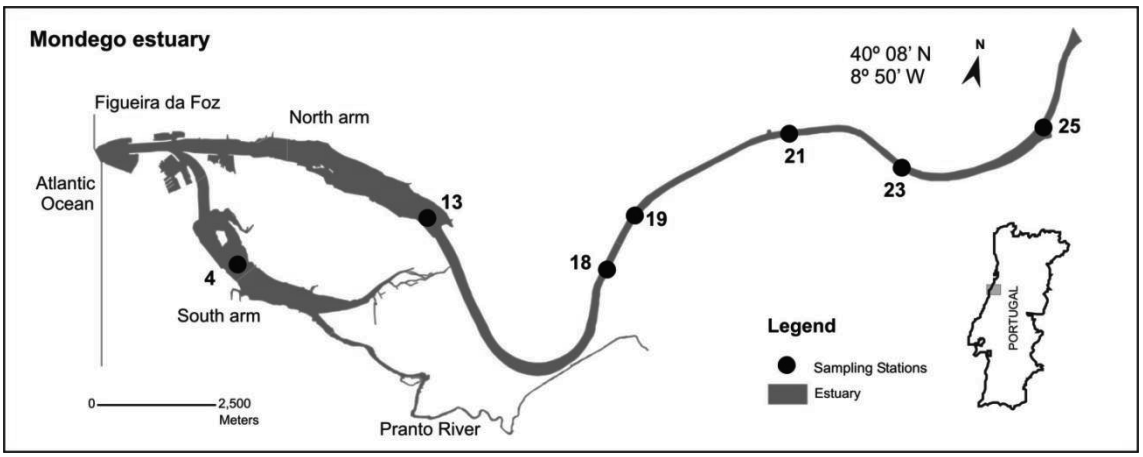


Figure 1. Mondego estuary (Portugal): station location (black circles).

Sampling strategy

In the summer of 2006, the subtidal soft-bottom assemblages (nematodes and macrofauna) were sampled at seven sampling stations (St4, St13, St18, St19, St21, St23, and St25), located along the north and south arms of the Mondego estuary (Fig. 1). The sampling stations were previously classified according to one of the five Venice salinity classes (Venice System, 1959): freshwater < 0.5 (St25), oligohaline 0.5–5 (St21 and St23), mesohaline >5–18 (St18 and St19), polyhaline >18–30 (no station), and euhaline >30 (St4 and St13), according to information gathered in previous studies (Teixeira et al., 2008).

Environmental data

Simultaneous with the sampling of the benthic invertebrates, the salinity, temperature (ºC), pH, and dissolved oxygen (DO) (mg L^{-1}) of the bottom water were measured *in situ*, and the Secchi depth recorded. Additionally, water samples were collected for measurement of nitrate (NO_3^--N) (µmol L^{-1}) and nitrite (NO_2^--N) (µmol L^{-1}), ammonium (NH_4^+-N) (µmol L^{-1}), and phosphate (PO_4^{3-}-P) (µmol L^{-1})

concentrations, and subsequently analyzed in the laboratory according to standard methods as described in Strickland and Parsons (1972) and Limnologisk Metodik (1992).

Due to logistic limitations in operating the sampling devices, subtidal sediment samples were collected at two levels. Thus, nematodes were collected close to the riverbank, at a depth of 1 m from the low-tide level ("M"), whereas macrofauna samples were obtained from the middle of the channel ("C") (at a depth ranging from 2.2 to 5.5 m at high tide conditions). Sediment organic matter (OM) content was defined as the difference between the weight of each sample after oven-drying at 60ºC for 72 h followed by combustion at 450ºC for 8 h, and was expressed as the percentage of the total weight. Grain size was analyzed by dry mechanical separation through a column of sieves of different mesh sizes, corresponding to the five classes described by Brown and McLachlan (1990): (a) gravel (>2 mm), (b) coarse sand (0.500–2.000 mm), (c) mean sand (0.250–0.500 mm), (d) fine sand (0.063–0.250 mm), and (e) silt and clay (<0.063 mm). The relative content of the different grain-size fractions was expressed as a percentage of the total sample weight.

Meiofauna and nematode assemblages

At each station, three replicates were collected by forcing a "Kajak" sediment corer (4.6 cm inner diameter) 3 cm into the sediment. All samples were preserved in a 4% buffered formalin solution. Meiofauna was extracted from the sediment fraction using Ludox HS-40 colloidal silica at specific gravity 1.18 g cm^{-3} and using a 0.038 mm sieve (Heip et al., 1985). All meiobenthic organisms were counted and identified at a higher taxonomic level under a stereomicroscope (magnification 40×). The abundance (individuals per 10 cm^2) of each meiofauna group was quantified. Meiofauna taxa identification was based on Higgins and Thiel (1988) and Giere (1993). A random set of 120 nematodes, or the total content of individuals in samples with less than 120 nematodes, was picked from each replicate. The nematodes were cleared in glycerol–ethanol solution, stored in anhydrous glycerol, and mounted on slides for identification (Vincx, 1996).

According to the majority of the meiobenthologists, nematode genus is considered a taxonomic level with good resolution to discriminate disturbance effects (Warwick, 1988a; Warwick et al., 1990; Gyedu-Ababio et al., 1999; Schratzberger et al., 2004; 2008; Moreno et al., 2008). Moreover, colonizer–persister (c–p) values allocated to marine and brackish nematodes used to calculate the Maturity Index (Bongers et al., 1991) were based on family and genus taxonomic level resolution. Therefore, nematode genera were identified according to the criteria of Platt and Warwick (1988), Warwick et al. (1998) and Eyualem-Abebe et al. (2006),

Macrofauna assemblages

Samples consisting of five replicates were removed using a Van Veen grab (model LMG) with an area of 0.078 m^2. Samples were sieved *in situ* through a 0.5 mm mesh sieve bag and preserved in a 4% buffered formalin solution. The collected specimens were later counted and identified at the species level, whenever possible.

Data analysis

Environmental variables

Environmental variables were square-root transformed (except dissolved oxygen and pH) whenever data were moderately skewed in distribution. All variables were then normalized and subjected to principal components analysis (PCA) for ordination. A lower triangular Euclidean distance matrix relating to the ordination was constructed (Clarke and Green, 1988). Two PCA analyses were performed, using the environmental parameters registered in the two subtidal levels ("M" where nematodes were collected, "C" were macrofauna was sampled).

The relationships between multivariate community structure and environmental variables were examined using the BIOENV procedure (Clarke and Ainsworth, 1993), which calculates rank correlations between a similarity matrix derived from biotic data and matrices derived from various subsets of environmental variables, thereby defining suites of variables that 'best explain' the

biotic structure. Environmental data were analyzed prior the BIOENV procedure in order to exclude highly correlated environmental variables. For the analyses of environmental variables, only one sample was taken from each station; therefore, the species abundances based on the number of replicates at each station were averaged for analyses linking biotic and abiotic data. Bray–Curtis similarity matrices, derived from the averaged transformed biotic data, were compared with the environmental distance.

Benthic fauna

Univariate analysis of the data

One-way ANOVA with "space" as the fixed factor (7 levels: St4, St13, St18, St19, St21, St23, and St25) was used to test for spatial differences with respect to total density, number of species, Margalef index (d), and Shannon–Wiener index (H'). Nematodes assemblages were analyzed using GMAV5 software (Institute of Marine Ecology, University of Sydney), after checking the homogeneity of the variance with the Cochran test. When differences were found, *a posteriori* comparisons were made using the Student–Newman–Keuls (SNK) test (Underwood and Chapman, 1997). The Kruskal–Wallis test was used to analyze spatial differences regarding nematode total density. For macrofauna communities, the analyses were carried out using the software package Minitab version 12.2. The data were checked for normality using the Kolmogorov–Smirnov test, and the homogeneity of variances was assessed using Levene's test. Data not meeting the homoscedasticity assumption were transformed.

Pair-wise differences were assessed with the post-hoc Tuckey test. Univariate measures were calculated for each sampling station based on the benthic invertebrate density data of all replicates, using the PRIMER 6.0 software package. To estimate the correlation between number of nematode genus, number of macrofauna taxa, nematode total density, macrofauna total density, d and H' for nematode, d and H' for macrofauna, MI (Maturity Index), ITD (Index of Trophic Diversity) and BAT (Benthic Assessment Tool), the Spearman correlation coefficient was calculated, using the Statistica 7 software package.

Multivariate analysis of benthic fauna data

Both for nematodes and for macrofauna communities, multivariate analysis was applied according to the procedures described by Clarke (1993), using the PRIMER version 6.0 software package (Clarke and Warwick, 2001) (Plymouth Marine Laboratory, UK). Lower triangular similarity matrices were constructed using square-root transformation and the Bray-Curtis similarity measure. Contributions to similarity by abundant species were reduced by transformations, and the importance of less-abundant species in the analyses thereby increased. ANOSIM was carried out to test for differences among estuarine stretches. Ordination was by non-metric multidimensional scaling (nMDS) (Kruskal and Wish, 1978; Clarke and Green, 1988). Taxa with the greatest contribution to differences between stretches of the estuary were identified using the similarity percentage analysis procedure (SIMPER) (cut-off percentage: 85%).

Ecological quality status assessment

Nematodes

The Maturity Index (MI, Bongers et al., 1991) was calculated to measure the impact of disturbances and to monitor changes in the structure and functioning of nematodes assemblages. Based on their specific characteristics, all nematode genera were distributed along a colonizer-persister (c-p) scale. The MI was calculated as the weighted mean of the individual taxon scores:

$$MI = \sum_{i=1}^{n} v(i).f(i)$$

where $v(i)$ = the c-p value of the taxon *i* (Table 1) and $f(i)$ = the frequency of that taxon. The index is expressed as a c-p value, ranging from c-p=1 for a colonizer to c-p=5 for a persister, and represents the life-history characteristics associated with r- and *K*-selection, respectively. Thus, taxa with *c–p* = 1 (colonizers) are *r*-selected, with short generation times, large population fluctuations, and high fecundity while taxa with *c–p* = 5 (persisters) are *K*-selected, producing few offspring and generally appearing later in a given succession (Bongers and Bongers, 1998; Bongers and Ferris, 1999). Low *c–p* values correspond to taxa that are relatively

tolerant of ecological disturbances, unlike taxa with high c-p values, which are sensitive (Neher and Darby, 2009). The MI, in practice, varies from 1, under extremely enriched conditions, to 3 or 4 under undisturbed conditions.

The Index of Trophic Diversity (ITD, Heip et al., 1985) was also estimated. Nematode genera were classified according to the criteria of Wieser (1953) into four feeding groups to investigate the trophic structure of the assemblage (Table 1): selective (1A) and non-selective (1B) deposit feeders, epistrate-feeders (2A), and predators/omnivores (2B). The ITD was then calculated as:

$$ITD = \sum \theta^2$$

where θ is the density contribution of each trophic group to total nematode density (Heip et al., 1985), ranging from 0.25 (highest trophic diversity, i.e., each of the four trophic guilds account for 25% of the nematode density) to 1.0 (lowest diversity, i.e., one trophic guild accounts for 100% of the nematode density).

Macrofauna

The Benthic Assessment Tool (BAT) (Teixeira et al., 2009), developed for soft-bottom benthic macrofauna, integrates, in a multimetric approach, three widely used metrics: the Shannon-Wiener diversity index, the Margalef index, and the AZTI Marine Biotic Index (AMBI). BAT values measure ecological quality along a scale from 0 (bad) to 1 (high). According to the method of Teixeira et al. (2009), the Ecological Quality Ratio (EQR) thresholds for defining ecological quality status (EQS) classes were used: 0-0.27 bad, 0.28-0.44 poor, 0.45-0.58 moderate, 0.59-0.79 good, and 0.80-1 high (for details regarding the index calculation, see Teixeira et al., 2009).

RESULTS

Environmental variables

Water transparency, DO, and salinity increased from the upstream stretch towards the mouth along both arms of the estuary (Table 2). The pH values were

similar throughout the system. The concentrations of nitrates and phosphates in the bottom water were, to some extent, spatially heterogeneous but, in general, were higher in the upstream stretch and decreased towards the mouth. Sediments in the "M" level of the estuary's upper stretches had a higher OM content than in the "C" level, wherein the OM content was essentially the same on average, regardless of the stretch. In the upstream stretch of the estuary, sediments from the "C" level consisted mostly of mean and coarse sand, while sediments of "M" level were very variable in particle-size composition.

The two ordinations of environmental factors determined by PCA allowed the different sampling stations to be categorized in four groups (Fig. 2): (1) freshwater, (2) oligohaline, (3) mesohaline, and (4) euhaline. Based on data from the environmental parameters, PCA showed that the first two principal components accounted for 87% of the total variability in the case of the M level (nematodes), and 90% in the case of the C level (macrofauna). In both analyses, variability along the first axis was mainly explained by an increase in temperature and in the concentration of nitrates, nitrites, ammonium, and phosphates from the mouth to the inner stations of the estuary, and a concomitant decrease of salinity and dissolved oxygen values. Variability along the second axis was mainly explained by the contrast between stations, i.e., stations characterized by higher proportions of fine sand, silt + clay, and OM *vs*. those with higher proportions of coarser sediments. In general, analogous ordinations were observed at both location levels.

Nematode assemblages

Table 3 shows the mean density (number of individuals per 10 cm^2) of meiofauna main taxa in each station. Although the proportion of nematodes decreased in the freshwater section, thus presenting a similar pattern to that observed in several other estuaries (Smol et al., 1994; Soetaert et al., 1994; 1995; Udalov et al., 2005), nematodes were the dominant taxon along the estuarine gradient representing 88% of the total meiofauna in the estuary. For this reason and because the more commonly used meiobenthic indicators use nematode data, from here after, the study was focused only on this phylum.

Table 1. c-p values (Bongers et al., 1991; Bongers, 1999), trophic group (Wieser, 1953) and total abundance (ind 10 cm^{-2}) for each of the nematode genera identified.

Taxa	c-p value	Trophic group	Total ind $10cm^{-2}$	Taxa	c-p value	Trophic group	Total ind $10cm^{-2}$
Metachromadora	2	2B	420.08	*Araeolaimus*	3	1A	2.14
Anoplostoma	2	1B	297.35	*Aponema*	3	1A	2.14
Daptonema	2	1B	214.56	*Paracomesoma*	2	1B	1.39
Sabatieria	2	1B	212.61	*Cromadorella*	3	2A	1.18
Microlaimus	2	2A	176.36	*Stygodesmodora*	3	2B	1.11
Sphaerolaimus	3	2B	92.99	*Doliolaimus*	3	2B	1.05
Axonolaimus	2	1B	82.18	*Paramonhystera*	2	1B	1.03
Prochromadorella	2	2A	61.53	*Spilophorella*	2	2A	1.03
Dichromadora	2	2A	60.14	*Tripyloides*	2	1B	0.77
Viscosia	3	2B	56.67	*Marylynnia*	3	2A	0.71
Paracyatholaimus	2	2A	56.10	*Paracanthoncus*	2	2A	0.64
Terschellingia	3	1A	45.18	*Monhystera*	2	1B	0.64
Leptolaimus	2	1A	43.28	*Valvaelaimus*	2	2A	0.43
Calyptronema	4	2B	34.97	*Odontophora*	2	1B	0.42
Chromadora	3	2A	30.59	*Comesoma*	2	1B	0.34
Paralinhomoeus	2	1B	30.40	*Diplolaimella*	1	1B	0.30
Aegialoalaimus	4	1A	26.50	*Syringolaimus*	4	2B	0.23
Linhomoeus	2	2A	23.93	**Freshwater nematodes**			
Halalaimus	4	1A	19.12	*Mesodorylaimus*	4	2B	80.37
Southerniella	3	1A	13.02	*Mononchus*	4	2B	4.11
Ptycholaimellus	3	2A	11.45	Ordem Aphelencida	2	-	1.81
Camacolaimus	3	2A	9.90	*Mylonchulus*	4	2B	1.36
Praeacanthoncus	4	2A	8.98	*Hemicycliophora*	3	-	1.20
Hypodontolaimus	4	2A	8.72	*Criconemella*	4	-	0.50
Chromadorita	3	2A	8.67	*Eutobrilus*	3	2B	0.34
Ascolaimus	2	1B	6.47	*Ironus*	4	2B	0.34
Chromadorina	3	2A	4.95	*Aphelenchoides*	2	-	0.33
Desmolaimus	2	1B	4.55	*Protodorylaimus*	4	2B	0.23
Oncholaimellus	3	2B	3.53	Ordem Rhabditida	1	1A	0.23
Cobbia	3	2A	2.40	*Laimydorus*	4	2B	0.21
Eumorpholaimus	2	1B	2.14	*Brevitobrilus*	3	2B	0.21

The colonizers-persistents scale (c-p value) is composed of five classes, 1 – 5; the colonizers, characterized by a high reproduction, receive a low value, the persistents, which reproduce slowly, are allocated to cp–5. Trophic Group: (1A) no buccal cavity or a fine tubular one - selective deposit (bacterial) feeders; (1B) large but unarmed buccal cavity - non-selective deposit feeders; (2A) buccal cavity with scraping tooth or teeth - epistrate (diatom) feeders; (2B) buccal cavity with large jaws - predators/omnivores.

Table 2. Environmental variables measured at each sampling station in the summer of 2006.

St	Transp. (m)	T (ºC)	DO (mg/l)	Sal	pH	$P\text{-}PO_4^{3-}$ (µmol/l)	$N\text{-}NO_3^-$ (µmol/l)	$N\text{-}NO_2^-$ (µmol/l)	$N\text{-}NH_4^+$ (µmol/l)	OM (%)		Gravel (%)		Coarse sand (%)		Mean sand (%)		Fine sand (%)		Silt+Clay (%)	
										M	C	M	C	M	C	M	C	M	C	M	C
4	3.2	17,6	8.7	32.2	7.9	0.96	14.68	0.16	0.99	0.9	0.7	1.6	7.9	7.9	49.5	27.6	38.6	60.9	3.9	2.0	0.1
13	2.8	17,8	8.8	31.8	7.8	0.82	3.12	0.14	0.93	1.4	0.5	29.7	9.4	26.3	23.8	22.0	63.5	17.5	3.2	4.5	0.0
18	1.1	22,1	7.3	18.5	7.5	1.54	26.28	0.78	1.99	4.8	0.3	1.1	19.7	11.4	65.5	16.2	14.2	59.1	0.6	12.2	0.0
19	1.1	22,1	7.5	15.2	7.4	1.64	29.95	0.88	1.92	3.8	0.4	0.2	10.4	0.9	71.5	14.4	16.7	74.1	1.2	10.4	0.2
21	0.7	22,8	6.3	5.5	7.2	1.98	50.63	1.50	2.32	3.0	0.6	38.4	3.2	1.7	58.1	15.9	34.5	39.0	3.8	5.1	0.4
23	0.7	23,6	6.2	0.1	7.3	2.99	97.68	3.28	3.01	4.1	0.3	8.8	21.1	3.1	69.0	16.9	9.3	64.4	0.5	6.7	0.1
25	0.6	23,9	6.5	0	7.4	2.94	95.15	4.22	4.49	0.2	0.3	35.8	17.3	46.0	69.0	16.2	12.2	1.9	1.3	0.2	0.2

St, station; Transp, transparency; T, temperature; DO, dissolved oxygen; Sal, salinity; $P\text{-}PO_4^{3-}$, phosphate; $N\text{-}NO_3^-$, nitrate; $N\text{-}NO_2^-$, nitrite; $N\text{-}NH_4^+$, ammonium; OM, sediment organic matter; gravel (>2 mm); coarse sand (0.5-2.0 mm); mean sand (0.25-0.50 mm); fine sand (0.063-0.250 mm); silt+clay (<0.063 mm); M, near the margin, 1 m depth from low-tide level; C, middle of the channel. (T, DO, Sal, pH, and nutrient concentrations were measured in the bottom water)

Sixty-one genera of nematodes belonging to 24 families were identified. The dominant families were Desmodoridae, Anoplostomatidae, Xyalidae, Comesomatidae, Chromadoridae, and Microlaimidae. The genera *Metachromadora* (19.3%), *Anoplostoma* (13.7%), *Daptonema* (9.9%), *Sabatieria* (9.8%), *Microlaimus* (8.1%), *Sphaerolaimus* (4.3%), *Axonolaimus* (3.8%), *Mesodorylaimus* (3.7%), *Prochromadorella* (2.8%), *Dichromadora* (2.8%), and *Viscosia* (2.6%) together represented 80.8% of the total nematode density. The freshwater and oligohaline stretches of the Mondego estuary were characterized by the presence of freshwater nematodes (*Mesodorylaimus* and *Mononchus*), and the mesohaline section by high densities of *Anoplostoma*, *Daptonema* and *Viscosia*, while in the euhaline section, *Metachromadora*, *Anoplostoma* and *Microlaimus* predominated in the Southern arm and S*abatieria*, *Leptolaimus*, and *Dichromadora* in the Northern arm. The mean density varied from 38.6 ± 3.2 individuals (ind) 10 cm^{-2} at St25 to 1323.1 ± 63.8 ind 10 cm^{-2} at St4. The significant difference between stations (H = 12.95, 6 d.f., p=0.0438) (Fig. 3A) was explained by the high density values recorded at a single station (St4).

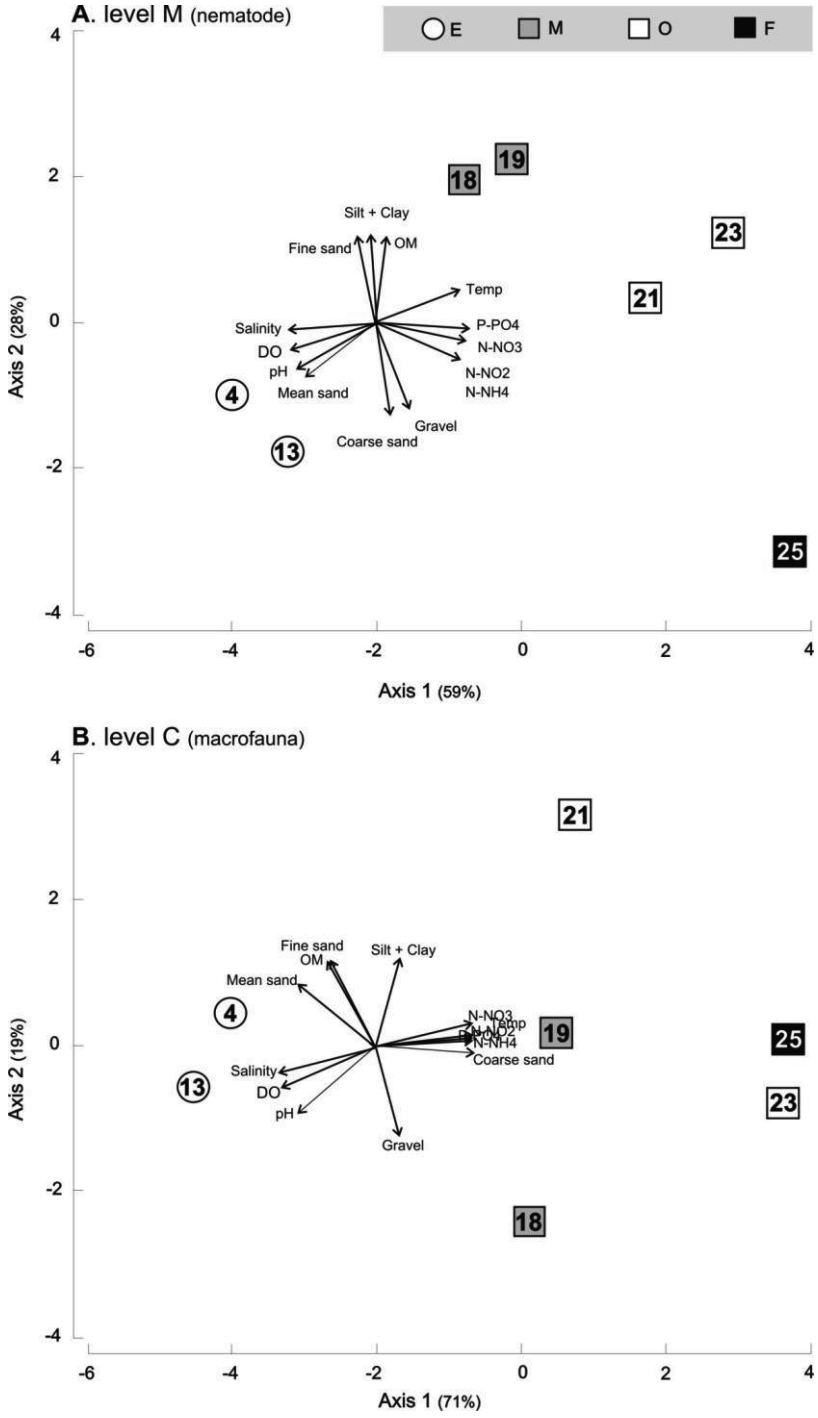


Figure 2. Principal Component Analysis (PCA) ordination of sampling stations and environmental variable vectors at the A) "level M" and B) "level C" of each stretch of the Mondego estuary. F, Freshwater; O, Oligohaline; M, Mesohaline and E, Euhaline.

Table 3. Mean density (number of individuals per 10cm^2) of meiofaunal taxa at each station in the Mondego estuary.

	St25	St23	St21	St19	St18	St13	St4
Nematoda	38.9	100.9	117.4	182.6	185.0	228.8	1323.1
Copepoda	3.0	1.0	0.6	0.4	4.0	6.8	30.9
Gastropoda	0.0	0.0	0.0	0.0	0.0	2.0	3.2
Ostracoda	0.2	0.0	0.0	1.0	1.4	0.0	4.0
Bivalvia	3.0	33.9	0.0	0.2	0.8	0.8	6.4
Polychaeta	37.5	34.1	15.9	46.6	81.1	24.1	4.8
Oligochaeta	0.0	0.0	1.4	1.0	0.0	1.2	4.0
Nauplii	0.4	0.2	0.0	0.0	0.0	0.6	5.2
Turbellaria	0.0	0.0	0.0	0.0	0.4	0.0	0.6
Amphipoda	0.6	0.0	0.0	0.2	0.2	0.0	0.8
Ciliophora	0.0	0.0	0.0	0.6	0.0	3.6	0.0
Cladocera	0.0	0.0	0.0	0.0	0.0	0.0	0.4
Halacaroidea	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Total	**83.7**	**170.2**	**135.3**	**232.6**	**273.1**	**267.9**	**1383.5**

There were significant differences between the stations regarding the number of taxa ($F_{6,14}$=3.40, p=0.03), with the lowest diversity (16 genera) detected at the oligohaline station (St23), and the highest (29 genera) in the euhaline stations (Southern arm). Among the latter, eight genera were found exclusively there (Fig. 3B). The only genus present in all sampling stations was *Daptonema*.

The Margalef index (Fig. 3C) did not significantly differ between the seven stations ($F_{6,14}$ =1.08; p=0.42), in contrast to the Shannon–Wiener index (Fig. 3D), which differed significantly between stations ($F_{6,14}$ = 8.19, p < 0.00062; SNK test p < 0.05), Specifically, the values at St4, in the euhaline area of the South arm, were significantly higher than those at St13, St18, St19, St23, and St25.

The ANOSIM test identified significant differences and thus distinct assemblages between the estuary's stretches (global R=0.804, p=0.001). The pair-wise test revealed significant differences between the assemblages from all stretches (p < 0.05). Significant results were also obtained for the oligohaline and mesohaline stretches (global R=0.37, p=0.009). Nevertheless, in those cases, the R-values differed only slightly between the groups, screening a real difference that could not have occurred by chance in the absence of a group effect. Therefore, ecologically, these two communities are indeed slightly different from each other.

The nMDS plot clearly reflected the spatial distribution of nematodes along the estuarine gradient (Fig. 4A). As described above, the sampling stations are completely separated from each other, and the euhaline stations in the Southern and Northern arms can be separated based on the composition and density of their nematode populations.

SIMPER analysis showed maximum dissimilarity between assemblages from the freshwater and those from the euhaline stretches of the Southern (99.3%) and Northern (98.4%) arms. The freshwater estuarine stretch was mostly characterized by freshwater nematodes (*Mesodorylaimus* and *Mononchus*). The euhaline assemblages present in the two arms were clearly distinguishable (dissimilarity 84.8%), mainly due to the higher density of *Metachromadora*, *Microlaimus* and *Anoplostoma* in the Northern arm and of *Sabatieria*, *Leptolaimus* and *Dichromadora* in the Southern arm (Table 4A).

BIOENV analysis showed that a combination of four variables, i.e., the percentage of mean sand and the N-compounds $N–NO_3$, $N–NO_2^-$ and $N–NH_4^+$, accounted for around 92% of the variability within the nematodes assemblages.

Table 4. Species determined by SIMPER analysis as contributing the most to the similarity within salinity stretches for (A) nematodes and (B) macrofauna assemblages. Shaded boxes: percent similarity (bold) and the species that contributed to the similarity in each group. Non-shaded box, percent dissimilarity (bold) between salinity stretches and the species that contributed to the total dissimilarity (cut-off percentage: 85%). NA, North arm; SA, South arm.

A. Nematodes	**Euhaline NA st4**	**Euhaline SA st13**	**Mesohaline st18 and 19**	**Oligohaline st21 and 23**	**Freshwater st25**
Euhaline NA st4	**48.9%** *Sabatieria* *Leptolaimus* *Dichromadora* *Daptonema*				
Euhaline SA st13	**84.8%** *Metachromadora* *Microlaimus* *Anoplostoma* *Sabatieria* *Prochromadorella* *Sphaerolaimus* *Axonolaimus* *Paralinhomoeus* *Terschellingia* *Calyptronema* *Chromadora*	**51.2%** *Metachromadora* *Anoplostoma* *Microlaimus* *Sabatieria* *Prochromadorella* *Sphaerolaimus*			
Mesohaline st18 and 19	**79.9%** *Sabatieria* *Daptonema* *Anoplostoma* *Leptolaimus*	**85.2%** *Metachromadora* *Microlaimus* *Anoplostoma* *Sabatieria* *Prochromadorella* *Sphaerolaimus* *Daptonema* *Axonolaimus*	**37.5%** *Anoplostoma* *Daptonema* *Viscosia*		

Table 4 continues in the next page

		Paralinhomoeus *Chromadora* *Calyptronema*			Table 4 (cont.)
Oligohaline st21 and 23	**84.4%** *Sabatieria* *Mesodorylaimus* *Daptonema* *Leptolaimus* *Paracyatholaimus* *Dichromadora* *Terschellingia* *Viscosia*	**93.8%** *Metachromadora* *Microlaimus* *Anoplostoma* *Sabatieria* *Sphaerolaimus* *Prochromadorella* *Axonolaimus* *Mesodorylaimus* *Paralinhomoeus* *Viscosia* *Chromadora* *Calyptronema*	**74.1%** *Anoplostoma* *Daptonema* *Mesodorylaimus* *Paracyatholaimus* *Viscosia* *Dichromadora* *Leptolaimus*	**32.7%** *Daptonema* *Paracyatholaimus* *Mesodorylaimus* *Anoplostoma*	
Freshwater st25	**98.4%** *Sabatieria* *Mesodorylaimus* *Leptolaimus* *Daptonema* *Dichromadora* *Terschellingia*	**99.3%** *Metachromadora* *Microlaimus* *Anoplostoma* *Sabatieria* *Prochromadorella* *Sphaerolaimus* *Axonolaimus* *Paralinhomoeus* *Viscosia* *Chromadora*	**96.4%** *Anoplostoma* *Daptonema* *Mesodorylaimus* *Viscosia* *Dichromadora* *Leptolaimus*	**76.8%** *Mesodorylaimus* *Daptonema* *Paracyatholaimus* *Anoplostoma* *Dichromadora* *Axonolaimus* *Mononchus* *Ascolaimus* *Leptolaimus*	**69.1%** *Mesodorylaimus* *Mononchus*
B. Macrofauna	**Euhaline NA st4**	**Euhaline SA st13**	**Mesohaline st18 and 19**	**Oligohaline st21 and 23**	**Freshwater st25**
Euhaline NA st4	**36.4%** Oligochaeta *Hydrobia ulvae* *C. glaucum* *Cerastoderma edule*				

Table 4 continues in the next page

Table 4 (cont.)

	Ophelia neglecta				
Euhaline SA st13	**81.6%** *Hydrobia ulvae* *C. glaucum* *Cerastoderma edule* *Cerastoderma* sp. Oligochaeta *Capitella capitata*	**53.9%** *Hydrobia ulvae* *C. glaucum* *Cerastoderma edule*			
Mesohaline st18 and 19	**95.3%** *C. multisetosum* *Streblospio shrubsolii* *Cyathura carinata* *Corbicula fluminea* *Cerastoderma edule* *C. glaucum* Oligochaeta	**98.7%** *Hydrobia ulvae* *C.multisetosum* *Streblospio shrubsolii* *C. glaucum* *Cyathura carinata* *Cerastoderma edule* *Corbicula fluminea*	**34.21%** *Streblospio shrubsolii* *C. multisetosum* *Corbicula fluminea* *Cyathura carinata*		
Oligohaline st21 and 23	**97.1%** *C. multisetosum* *Corbicula fluminea* *Cyathura carinata* *Cerastoderma edule* *C. glaucum* Oligochaeta *Hydrobia ulvae*	**99.3%** *Hydrobia ulvae* *C.multisetosum* *C. glaucum* *Corbicula fluminea* *Cerastoderma edule* *Cyathura carinata* *Cerastoderma* sp.	**77.1%** *C. multisetosum* *Streblospio shrubsolii* *Corbicula fluminea* *Cyathura carinata*	**49.4%** *C. multisetosum* *Corbicula fluminea* *Cyathura carinata*	
Freshwater st25	**98.2%** *Corbicula fluminea* *C. multisetosum* *Cerastoderma edule* *C. glaucum*	**99.2%** *Corbicula fluminea* *Hydrobia ulvae* *C. glaucum* *Cerastoderma edule* *C. multisetosum*	**86.6%** *Corbicula fluminea* *C. multisetosum* *Streblospio shrubsolii*	**63.4%** *Corbicula fluminea* *C. multisetosum* *Cyathura carinata*	**80.8%** *Corbicula fluminea*

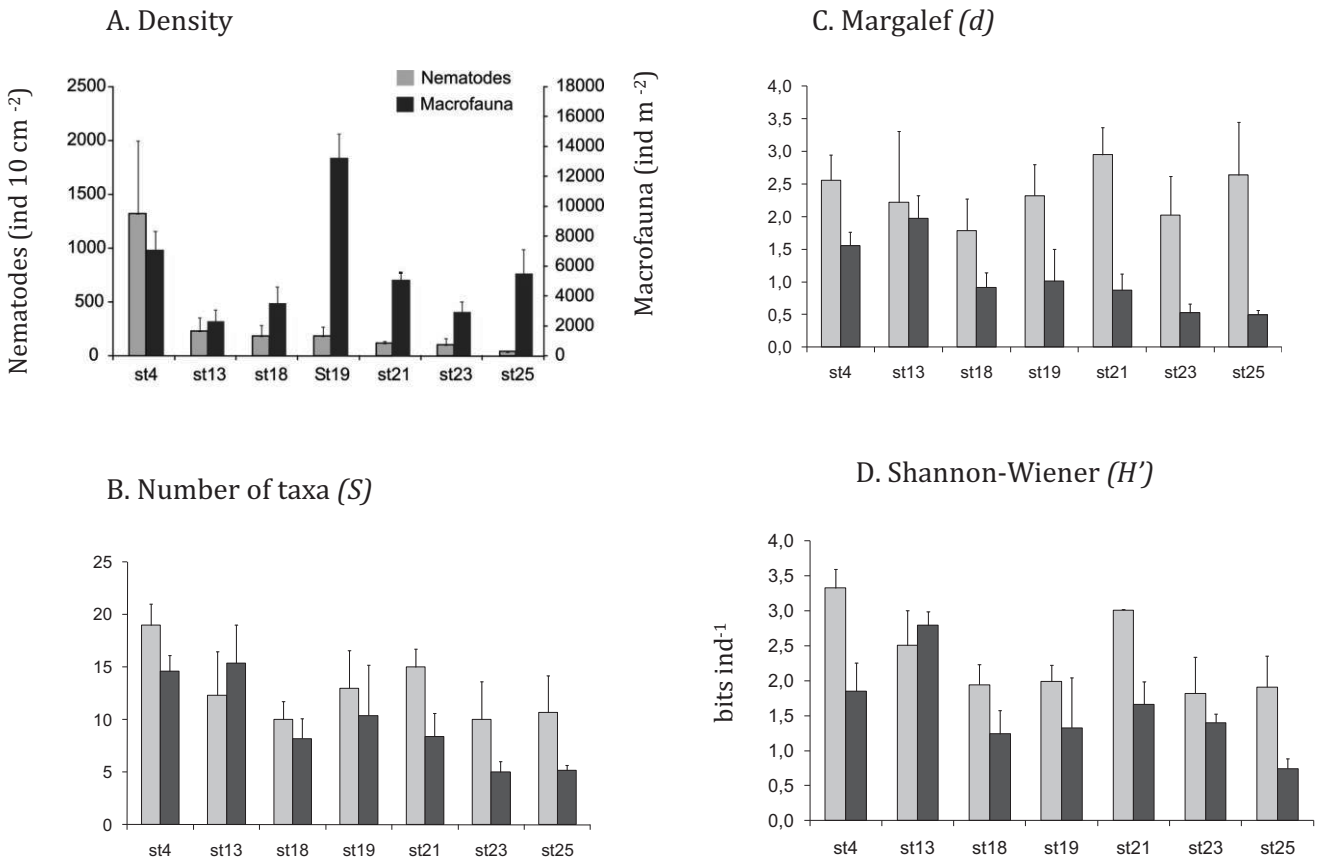


Figure 3. Nematodes and macrofauna. (A) Mean density ± SD (ind 10 cm^{-2}, ind m^{-2}, respectively); (B) Number of taxa; (C) Margalef index; (D) Shannon-Wiener index (bits ind^{-1}) observed at each sampling station.

Macrofauna assemblages

Of the 105 macrofauna taxa identified along the estuary, 92.9% of the total macrofaunal density was accounted for by: *Corophium multisetosum* (33.8%), *Corbicula fluminea* (20.5%), *Hydrobia ulvae* (11.3%), *Cyathura carinata* (10.1%), *Streblospio shrubsolii* (8.1%), *Cerastoderma glaucum* (3.7%), *Cerastoderma edule* (3.2%), and Oligochaeta (2.2%).

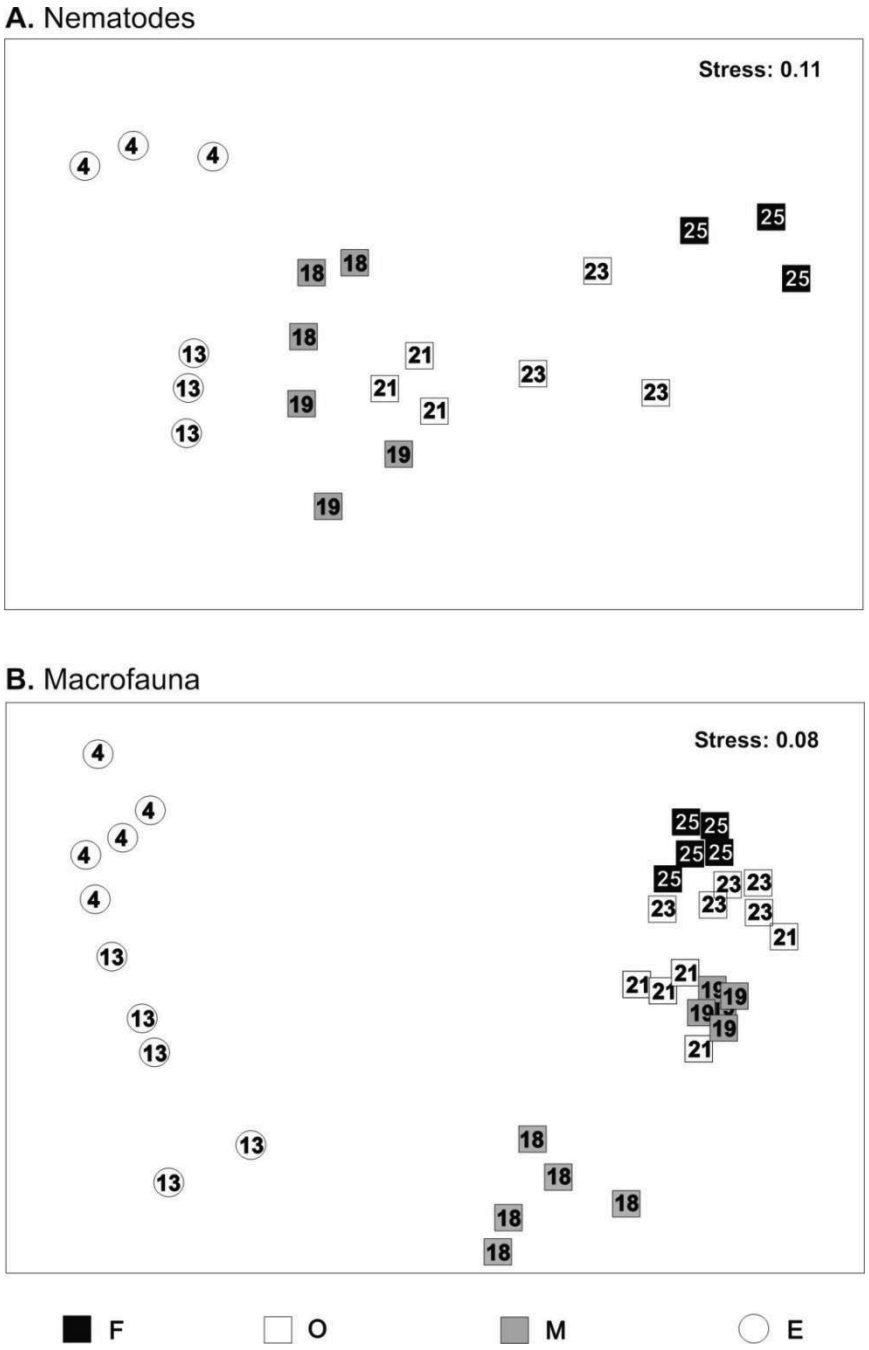


Figure 4. Non-metric multidimensional scaling (nMDS) ordination plots of root-transformed faunal abundance data comparing (A) nematode and (B) macrofauna community structures at each sampling station. Numbers indicate stations and symbols indicate stretches

The mean density varied between 1774 ± 1297 ind m^{-2} at St13 and 12717 ± 2143 ind m^{-2} at St19. Significant differences in macrofaunal density were recorded

between stations ($F_{6,28}$=17.94, p=0.0001) (Fig. 3A). The mean density at St19 was significantly higher than at all other stations with the exception of St4. This last station had significantly higher values than at St13, St18, and St23. The number of species differed significantly between stations ($F_{6,28}$=24.09, p=0.0001) (Fig.3B), with a higher number of species present at the euhaline stations than at all stations with the exception of St19, where the number was significantly higher than that determined at either St18 (belonging to the same mesohaline area) or the oligohaline and freshwater stations.

Regarding the Margalef index (Fig. 3C), unlike the case for nematodes, significant differences were found between the seven stations ($F_{6,28}$=32.65, p=0.0001), with a higher species richness again recorded at the euhaline stations than at all the other estuarine stations. The values obtained at mesohaline St19 were significantly higher than those of the two most upstream stations (St23 and St25). The Shannon-Wiener index (Fig 3D) was also significantly different between stations ($F_{6,28}$=23.97, p=0.0001), with significantly higher values at St13, located in the North arm than at all other stations. Furthermore, the values at the freshwater station (St25) were significantly lower than those at St4 St19, St21, and St23.

The ANOSIM test showed highly significant differences and thus distinct assemblages between estuarine stretches (global R=0.694, p = 0.001). Moreover, the pair-wise tests indicated significant differences among all of the assemblages ($p < 0.05$). The results were confirmed by the nMDS plot (Fig. 4B).

As with nematodes, the euhaline stretch was divided in terms of the Northern and Southern arms in order to capture possible differences between these two subsystems (Table 4B). The results showed high levels of dissimilarity between the assemblages from the different salinity stretches, with the dissimilarity between the euhaline stations of the two arms and those of the mesohaline, oligohaline and freshwater stretches ranging between 95% and 99%. Both euhaline areas were mainly characterized by *H. ulvae* and *Cerastoderma* sp. Variations in the relative abundance of these common species accounted for most of the dissimilarity between the two euhaline subsystems (higher values in the Southern arm). The assemblages of the mesohaline stretches were characterized by high abundances of *C. multisetosum*, *C. carinata*, *S. shrubsolli* and *C. fluminea*. It

was interesting to note that *C. multisetosum* and *C. fluminea* showed impressive abundances around this salinity stretch (4022 ind m^{-2} and 700 ind m^{-2}, respectively). These two species were also characteristic of the freshwater stretch (1712 ind m^{-2} and 4228 ind m^{-2}, respectively).

BIOENV analysis identified salinity and DO as the most relevant variables explaining the macrofaunal spatial pattern ($\rho = 0.83$).

Ecological quality status assessment

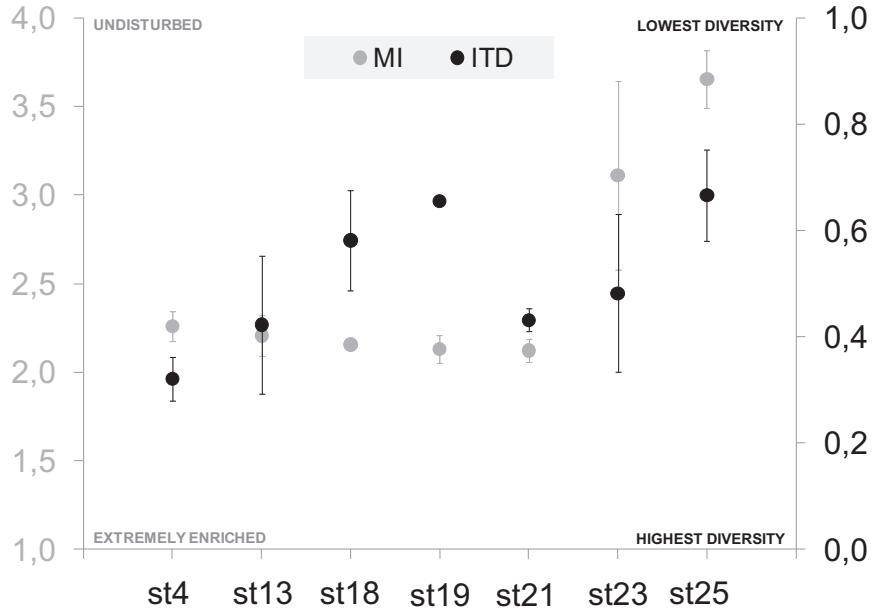
Nematodes

The ITD clearly discriminated between nematode assemblages belonging to each estuarine stretch, with the highest trophic diversity occurring at the euhaline stations. At the freshwater station, the ITD was relatively high (low diversity) mainly due to the dominance of "predators/omnivores" (2B) (Fig 5A). By contrast, the MI values were similar between most sampling stations, only differentiating the upstream stretch from the other stretches. The highest values were recorded at St23 and St25, where the conditions were undisturbed, as defined by Bongers et al. (1991).

Macrofauna

The BAT results showed that the EQS ranged from 'Poor' to 'Moderate' (Fig 5B). The lowest quality was found in the freshwater stretch (St25) and the highest in the oligohaline area. Although the values obtained for the mesohaline stations were within the classification range determined for the other stations, the within-site variability was higher (particularly at St18).

A. MI and ITD



B. BAT

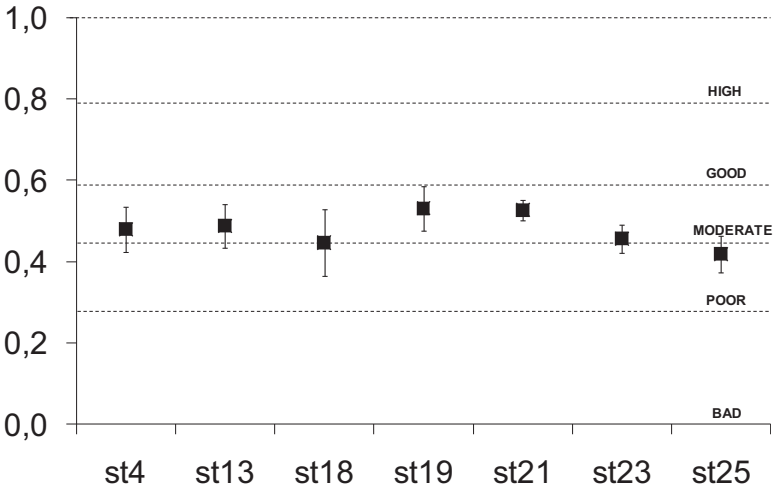


Figure 5. Spatial changes at each sampling station in: (A) the Maturity Index (MI) and Index of Trophic Diversity (ITD) and (B) the BAT.

DISCUSSION

As mandated by the WFD, existent aquatic ecosystems with natural gradients arising from differences in salinity, particles size, organic matter content, nutrients, sediment cover, etc., must be surveyed. However, only a few of the field studies that examined the spatial distribution patterns also compared, directly and simultaneously, changes occurring in macrofaunal and nematode assemblages in response to such gradients. Although lacking temporal replication, our survey provides an assessment of the current ecological conditions in an estuarine system, thus providing a baseline for the future monitoring of long-term changes by examining their effects on these two different benthic invertebrate communities.

Influence of environmental factors

Due to logistic constrains, nematode and macrofauna assemblages have been sampled at two different depth levels (and probably in different microhabitats) within the same river stretch. Although the environmental variables measured along the Mondego estuary clearly reflected an estuarine gradient ranging from freshwater to euhaline areas, specifically, in terms of salinity, particle size, and nutrients in the water, the abiotic complementary data also showed within-level differences. These changes may have contributed to affect the small-scale response of the assemblages to other super-parameters such as the aforementioned ones. In addition, two gradients were clearly recognizable in the North and South arms of the estuary, which can be explained by their distinct hydrological regimes. BIOENV analysis showed that the distribution of nematode and macrofaunal communities can be explained by distinct environmental factors. The main structuring factors for nematode were the nutrient concentration in the estuary's waters and grain size. The prime importance of the estuarine gradient structuring the spatial distribution, abundance and species composition of free-living nematodes has been described in several other studies as well (Austen and Warwick, 1989; Vincx et al., 1990; Coull, 1999; Ferrero et al., 2008; Adão et al., 2009). For macrofaunal communities, the primary structuring factors were probably differences in salinity

and DO, characteristic of transitional systems (Bulger et al., 1993; Attrill, 2002; McLusky and Elliott, 2004). Thus, whereas several environmental parameters determined the structure of nematode assemblages, only two factors could affect the macrofaunal assemblages, suggesting that nematodes are more receptive to within-habitat physical variability than macrofauna (also observed by Schratzberger et al., 2008). In fact, the spatial patterns of temperate nematode communities on different horizontal scales have already been investigated extensively in different estuaries. Most of these studies related structural patterns of the nematode assemblages to environmental variables as sedimentary and latitudinal gradients, food resources, salinity and disturbances of different nature (Guo et al., 2001).

Community structure

Meiobenthos and macrobenthos communities, in addition to being separated on the basis of size, have a series of distinctive ecological and evolutionary characteristics suggesting that the segregation of the two groups is a meaningful one (Warwick, 1984). The small size, the high diversity and density of nematodes, associated with shorter generation times and no planktonic phase in their life cycles, allow (potentially) shorter response time (Gyedu-Ababio et al., 1999; Moens et al., 1999). Likewise, it can be expected that these two components of the benthos respond differently to disturbances of their communities, and that these responses provide an interesting and useful basis of comparison.

Number of taxa

In the Mondego estuary, nematode communities were made up of a high number of genera, but with few dominant taxa, as observed in other systems (Austen et al., 1989; Li and Vincx, 1993; Soetaert et al., 1995; Steyaert et al., 2003; Ferrero et al., 2008). As was the case for density, the number of genera tended to decrease, consistent with the transition from the sea to freshwater. This pattern was also found in studies of other European estuaries (Heip et al., 1985; Soetaert et al., 1995; Coull, 1999), although these environments were made up of fewer genera. A clear tendency of a decreasing number of taxa from euhaline to

freshwater areas was also observed for macrofauna communities. This pattern is abundantly described in the literature and corresponds to the Remane diagram, redrawn according to the two-ecocline model proposed by Attrill and Rundle (2002), in which freshwater species are shown to decrease as salinity increases, and marine species decrease as salinity decreases. Very few species, however, are physiologically adapted to survive in the salinity of the oligohaline zone (Dauvin and Ruellet, 2009).

Density and composition

Macrofauna and nematode densities changed along the estuarine gradient. Meiofaunal communities were clearly dominated by nematodes (Alves et al., 2009), which were of low density in the freshwater and oligohaline stretches of the estuary and of higher density in its euhaline stretches. This pattern was similar to those observed in several other estuaries (Smol et al., 1994; Soetaert et al., 1994, 1995; Udalov et al., 2005). Moreover, the density values were similar to those reported for the communities living in subtidal sediments of Northern European estuaries (Smol et al., 1994; Soetaert et al., 1994). Macrofaunal density differed in distribution, with the maximum density reached in the mesohaline stretch, due to the extremely high density of r-selected species such as *C. multisetosum*, followed by *C. carinata* and *S. shrubsolli*, and a minimum in the euhaline stretch.

The transition from freshwater fauna to typical estuarine assemblages and then to marine communities has been observed for both benthic groups. Particularly, regarding nematode, *Daptonema* was present along the entire Mondego estuary (this study) and the Thames estuary (Ferrero et al., 2008), reflecting the wide salinity tolerances known for many estuarine species (e.g. Heip et al., 1985).

In our study area, the two communities gave the same "picture" of the estuary and closely followed its estuarine gradient, with the distinction between stretches even more evident as represented by the nematode community. Given their small size and low mobility, nematodes are more susceptible to within-habitat physical variability than larger, more mobile, and potentially more highly dispersed members of the macrofauna (as described for polychaetes by

Schratzberger et al., 2008). As observed by Schratzberger et al. (2008) in two offshore subtidal habitats of the west coast of the UK, the similarity of the studied communities also significantly decreased with distance at the spatial scales sampled, with the trend being more evident in benthic nematodes. The number of microhabitats and niche speciation within seemingly homogenous sediments is high for nematode and this can result in high variability at small spatial scales (Schratzberger et al., 2008). Species respond to spatial variation in the environment at their own unique scales and this is function of their behaviour, body size, mobility and dispersal potential (Schratzberger et al., 2008).

Ecological assessment information

The objective of classical community indices is to condense community data into one or a few variables to simplify analysis, interpretation, or review (Neher and Darby, 2009). For the communities analyzed in the present study, the broadly used Margalef and Shannon–Wiener indices generally followed the number of taxa, with higher diversity and equitability in the euhaline stations. The lower Shannon–Wiener index values determined for stations 18 and 19 (mesohaline) suggested that at these sites both assemblages were under some type of stress (Gyedu-Ababio et al., 1999). However, whether the disturbances were natural, anthropogenic, or both could not be determined since the responses to the two types of stress are difficult to differentiate ("Estuarine Quality Paradox"; Elliott and Quintino, 2007).

In the broadest sense, diversity can refer to the sum of the differences imposed by life form and function, including multiple scales of organization, spatial arrangement (alpha, beta, and gamma diversity), habitat, and environmental disturbance (Neher and Darby, 2009). Current research is largely based on the description of assemblages using a taxonomic approach, but in ecology the coupling of taxonomic and functional diversity can also be a powerful tool. The functional role of nematodes in terms of feeding type, as first described by Wieser (1953), can be exploited to better understand the dynamics of a particular ecosystem, as this approach, despite its known limitations, yields insights into the system's mode of function. The relative proportion of each of the four nematode

feeding guilds in a community generally depends on the nature of the available food, which in turn is dependent on sediment composition (Moens and Vincx, 1997; Danovaro and Gambi, 2002). According to the ITD values, the trophic composition of the assemblages varied along the Mondego estuary but did not follow a regular pattern. At the freshwater station the ITD was relatively high (low trophic diversity), mainly due to the dominance of omnivores/predators whereas at the euhaline section trophic diversity was higher, with more even representation of all feeding groups.

Other authors (e.g. Gyedu-Ababio et al., 1999) suggested that a triad of metrics, the MI, Shannon–Wiener diversity index (H'), and the c–p (%), is a useful tool in pollution monitoring, especially organic pollution involving nematodes. For instance, Beier and Traunspurger (2001), studying two small German streams, noted that the MI decreased in polluted sites. At our study site, despite the differences in density, composition and structure along the estuary, the MI values in the mid-estuary and downstream sections were very similar, with 42% of the genera classified as colonizers (c–p=2). Nematodes with a c–p value of 2 are considered opportunistic and able to take advantage of disturbed or polluted environments (Gyedu-Ababio and Baird, 2006). However, the MI was not affected by the low diversity and density values of the freshwater and oligohaline sections and classified these areas as undisturbed. Comparing with Soetaert et al. (1995), where the meiofauna from the intertidal zone of five European estuaries (Ems, Westerschelde, Somme, Gironde, Tagus) covering various benthic habitats, from near-freshwater to marine and from pure silts to fine-sandy bottoms was investigated, we may see that the MI values determined for the Mondego estuary fall within those of other European estuaries ($2 < MI < 2.5$), with the exception of the freshwater station in the Gironde, where the index was much lower than at other stations.

According to the BAT results, the EQS varied between 'Poor' and 'Moderate,' with the lowest quality determined for the freshwater stretch. Although the BAT values of the mesohaline stations were within the classification range of the other stations, there was higher within-site variability (particularly at St18). Thus, the upstream classifications must be interpreted with caution, pending further

adjustment of the BAT's boundary values between thresholds of quality classes, in order to deal with natural gradients (Teixeira et al., 2009).

Overall, the results of our study allow us to answer the question whether nematode and macrofauna assemblages provide comparable ecological assessment information (Table 5) as follows:

Table 5. Summary of the trends revealed by the Margalef index (d), Shannon-Wiener diversity index (H'), Maturity Index (MI), Index of Trophic Diversity (ITD) and Benthic Assessment Tool (BAT) for each salinity stretch.

Stretch	Nematodes				Macrofauna		
	d	*H'*	MI	ITD	*d*	*H'*	BAT
Euhaline	+	+	+/-	+	+	+	+/-
Mesohaline	-	-	+/-	-	+/-	-	+/-
Oligohaline	+/-	+/-	+/-	+	+/-	+/-	+/-
Freshwater	+	-	+	-	-	-	-

(+) better ecological status; (+/-) intermediate ecological status; (-) lower ecological status

1. Euhaline stretch: In general, assemblages of the two benthic invertebrate groups in this area were rich in diversity and regularly structured. The ITD value confirmed this result, indicating high trophic diversity within the nematode community. By contrast, the MI values were low, reflecting the fact that they were characterized by a high percentage of colonizer taxa, typical of disturbed conditions. The BAT values were in line with the MI, classifying the EQS as moderate. Although located in different subsystems, the water conditions of St4 and St13 were similar to those in this stretch, differing essentially only with respect to sediment parameters (OM and granulometry). The sediment composition is very important for macrofauna, and for these two euhaline stations it might explain the disagreement between the BAT results and the Margalef and Shannon–Wiener results. The higher percentage of fine sediments and sediment OM can naturally favor the presence of organisms (e.g. polychaetes and oligochaetes), usually associated with more polluted areas. These differences in composition are described by the AMBI (it considers species sensitivity to organic enrichment), counterbalancing the results of the diversity indices and lowering the St4 score.

2. Mesohaline stretch: Here, the structural diversity of nematodes and macrofauna was low while the ITD values reflected the low trophic diversity. The MI and BAT values were in accordance with this stretch's moderate ecological quality status.

3. Oligohaline stretch: All indices described an intermediate classification compared to the other two stretches. The only exception was the ITD pertaining to the nematode assemblage, as its trophic composition was relatively diverse.

4. Freshwater stretch: While the ITD and BAT indicated low trophic diversity and poor ecological status, respectively, the MI values suggested the opposite, as in this area they were the highest, typical of undisturbed environmental conditions. The interpretation/integration of the classification results is far from being straightforward, particularly in the oligohaline/freshwater stretches. Strong water flow and bottom shear stress, together with low salinity values and high daily variations of water temperature, are often pointed out as factors that determine difficult conditions for macrofauna species' establishment and survival. Information on upper areas of transitional waters is scarce, although enough to conclude that we are in presence of an inhospitable environment that supports the least diverse communities or organisms found between freshwater and the sea (e.g. Remane and Schlieper, 1971; Jordan and Sutton, 1984). Therefore, it is really a challenge to distinguish between natural higher selective pressure and consequences of human-induced disturbance. In the Mondego estuary, these stretches are, in fact, characterized by a very low number of species and the assemblages are dominated by the exotic clam *C. fluminea* (Vinagre, personnal presentation). According to Phelps (1994) and Darrigran (2002), once established, this invasive species may have considerable ecological impacts such as changes in food webs and competition with native species. Specifically, in this study, we only found *C. fluminea*, *C. multisetosum*, Oligochaeta, *C. carinata*, Chironomidae larva, *Spio* sp. and *Gammarus* sp. So, we cannot say for sure that these species are the only able to cope with the high natural selectivity or that, instead, they are the only able to resist to *C. fluminea* competitive pressure or to other unidentified source of anthropogenic stress. BAT, a taxonomic sufficiency-based multimetric index, is telling us that the upstream areas are in "Poor" condition, reflecting the low

number of species and the presence of *C. fluminea* and the opportunistic oligochaete species. The question that has to be raised is: would these assemblages be different (e.g. higher diversity, without opportunistic species) in a pristine condition? Unfortunately, we are still not able to answer the question undoubtedly. On the other hand, nematode assemblages also showed a reduction in species number in the oligohaline/freshwater stretches. Besides, the fewer species, in general, according to MI, the species are persisters (life-history characteristics associated with K-selection) and the assemblage shows low trophic diversity (high ITD values). Are these indications of lower natural selectivity pressure on this benthic component? We cannot say definitely.

Thus, the answer to the question posed in the title of this paper appears to be difficult. Our results, more than giving clear patterns, left us with several unsolved challenges. Although both invertebrate groups were characterized by distinctive assemblages along the estuary, consistent with the estuarine stretches defined *a priori*, when several structural and functional attributes were analyzed in detail, differences between the two groups were revealed. Moreover, for each benthic group, in several respects the ecological indicators gave divergent information. For instance, ITD and MI are indicators of ecosystem function; the first focusing on the trophic structure of the assemblages and the second on the life strategy characteristics of nematodes. However, although applied to the same nematodes dataset, they yielded different classifications of the ecosystem. Moreover, this was also the case for the classical diversity indices. The uncertainty became even greater for the integration of macrofauna data. This finding highlights the need to develop a nematode-based multimetric index that takes into account abundance, composition and taxon sensitivity to stress (similar to the multimetric BAT for macrofauna), in order to provide clearer information regarding ecosystem status in accordance with the WFD requisites.

In summary, our study shows that macrofauna and meiobenthic nematodes may provide different but complementary types of information, depending on the indices used and the different “response-to-stress” times of each benthic group. Optimally, both groups should be used in marine pollution monitoring programs.

Chapter 2

Benthic meiofauna as indicator of ecological changes in estuarine ecosystems: The use of nematodes in ecological quality assessment

Benthic meiofauna as indicator of ecological changes in estuarine ecosystems: The use of nematodes in ecological quality assessment

ABSTRACT

Estuarine meiofauna communities have been only recently considered to be good indicators of ecological quality, exhibiting several advantages over macrofauna, such as their small size, high abundance, rapid generation times and absence of a planktonic phase. In estuaries we must account not only for a great natural variability along the estuarine gradient (e.g. sediment type and dynamics, oxygen availability, temperature, flow speed) but also for the existence of anthropogenic pressures (e.g. high local population density, presence of harbours, dredging activities).

Spatial and temporal biodiversity patterns of meiofauna and free-living marine nematodes were studied in the Mondego estuary (Portugal). Both taxonomic and functional approaches were applied to nematode communities in order to describe the community structure and to relate it with the environmental parameters along the estuary. At all sampling events, nematode assemblages reflected the estuarine gradient, and salinity and grain size composition were confirmed to be the main abiotic factors controlling the distribution of the assemblages.

Moreover, the low temporal variability may indicate that natural variability is superimposed by the anthropogenic pressures present in some areas of the estuary. The characterization of both meiofauna and nematode assemblages highlighted the usefulness of the integration of both taxonomic and functional attributes, which must be taken into consideration when assessing the ecological status of estuaries.

***Keywords*:** meiobenthos, free-living nematodes, indicators, biodiversity, estuaries.

INTRODUCTION

Meiofauna features are a good indicator of environmental conditions and changes in their density, diversity, structure and functioning may indicate alterations in the system. Although not being included in the biological compartment that needs to be monitored in the scope of the Water Framework Directive (WFD, Directive 2000/60/EC), meiofauna gives valuable information regarding ecosystems health. According to Sheppard (2006), marine scientists need to increase awareness of and emphasize the importance of the many species that have no appeal, which are not attractive and, for the most part, are not seen, like meiofauna.

Despite these difficulties, meiofauna communities are reasonably well characterized around the world, with studies ranging from the deep sea floor to alpine lakes, as well as from tropical reefs to polar sea ice (Giere, 2009). In Europe, studies on meiobenthic communities mostly encompass the more northerly estuarine ecosystems (e.g. Warwick and Gee, 1984; Li and Vincx, 1993; Smol et al., 1994; Soetaert et al., 1995, Ferrero et al., 2008). In southern Europe there is a serious gap in knowledge. Particularly in the Iberian Peninsula, there is a lack of information on both spatial and temporal distribution of meiofauna and free living nematodes in estuarine environments, being essential to describe those biodiversity patterns.

Meiobenthic communities provide information of great interest not only due to their important role in marine benthic food chains (Heip et al., 1985; Moens et al., 2005) but also due to their ecological characteristics (small size, high abundance, rapid generation times and absence of a planktonic phase), giving meiofauna several advantages over the commonly used macrofauna communities as monitoring organisms (Kennedy and Jacoby, 1999; Schratzberger et al., 2000; Austen and Widdicombe, 2006). In fact, nematodes have been pointed out as potential indicators of anthropogenic disturbance in aquatic ecosystems (e.g. Coull and Chandler, 1992; Schratzberger et al., 2004; Steyaert et al., 2007; Moreno et al., 2008). The inclusion of information regarding their functional traits (e.g. trophic

structure, life strategy) can provide critical information on the functioning of ecosystems (Norling et al., 2007; Danovaro et al., 2008).

Estuaries are naturally stressed systems with a high degree of variability in their physical-chemical characteristics. The natural gradient of salinity, linked with other gradients (e.g. bed sediment type and dynamics, oxygen availability, temperature and current speed), are well documented as important factors in determining temporal and spatial variations of meiofauna communities (Bouwman, 1983; Heip et al., 1985; Austen and Warwick, 1989; Soetaert et al., 1995; Li et al., 1997; Forster, 1998; Moens and Vincx, 2000; Steyaert et al., 2003; Derycke et al., 2007; Alves et al., 2009; Adão et al., 2009) but studies encompassing the entire salinity range from marine to freshwater conditions are few (e.g. Portugal: Alves et al., 2009; Adão et al., 2009; Patrício et al., 2012; United Kingdom: Ferrero et al., 2008; The Netherlands: Soetaert et al., 1994; Australia: Hourston et al., 2011). Moreover, most studies cover a small temporal range, providing only limited information on the behaviour of assemblages over longer time scales.

The present study compares the characteristics of meiofauna and free living nematodes assemblages in the subtidal sediments of different locations from Euhaline to Oligohaline areas of the Mondego estuary. Furthermore, the temporal (seasonal) variability between the assemblages of different locations is assessed and the use of nematodes as biological indicators of environmental quality is considered.

This study aimed to investigate changes in patterns of meiofauna and nematode assemblage composition and nematode diversity, trophic composition and life strategies between different estuarine locations and sampling occasions

The following null hypotheses were tested: a) There would be no differences in meiofauna taxon and nematode assemblage composition and trophic composition along the estuary; b) There would be no differences in the meiofaunal taxon and nematode assemblage composition and trophic composition at different seasonal sampling events.

MATERIALS AND METHODS

Study area

The Mondego estuary (Fig. 1), located on the Atlantic coast of Portugal (40º08'N, 8º50'W), is a polyhaline system influenced by a warm-temperate climate. The estuary is 21 km long (based on the extent of tidal influence) with an area of about 8.6 km^2 and, in its terminal part (at a distance of 7 km from the sea) it divides into two arms, northern and southern, separated by an alluvial island (Murraceira island), which rejoin near the estuary's mouth. The two arms have very different hydrological characteristics. The northern arm is deeper (5 - 10 m during high tide), receives most of the system's freshwater input, being influenced by seasonal fluctuation in water flow (Flindt et al., 1997), and forms the main navigation channel on which the Figueira da Foz harbour is located. The southern arm is shallower (2 - 4 m during high tide), has large areas of intertidal mudflats (almost 75% of the area) exposed during low tide and, until the spring of 2006, was almost silted up in the upper zones. In May 2006, the communication between both arms was re-established in order to improve the water quality in the terminal part of the estuary by reducing the residence time in the southern arm (Neto et al., 2010).

The Mondego estuary supports not only the Figueira da Foz harbour (regular dredging is carried out to ensure shipping conditions) but also numerous industries and receives agricultural run-off from rice and corn fields in the Lower River valley (Marques et al., 2003).

Sampling strategy

The subtidal soft-bottom meiobenthic assemblages were sampled along the salinity gradient of the Mondego estuary on six sampling occasions: August 2006 (summer, Su06), November 2006 (autumn, Au06), March 2007 (winter, Wi07), June 2007 (spring, Sp07), September 2009 (summer, Su09) and December 2009 (autumn, Au09).

Eleven sampling stations were selected following the division of the estuary proposed by Teixeira et al. (2008) (Fig. 1). The estuary was thus divided in five

different areas: Euhaline (station 4; salinity 30-34); Polyhaline of the South Arm (st 6, 7 and 9; salinity 18-30), Polyhaline of the North Arm (st 12 and 13; salinity 18-30), Mesohaline (18 and 19; salinity 5-18) and Oligohaline (st 21, 23 and 25; salinity 0.5-5).

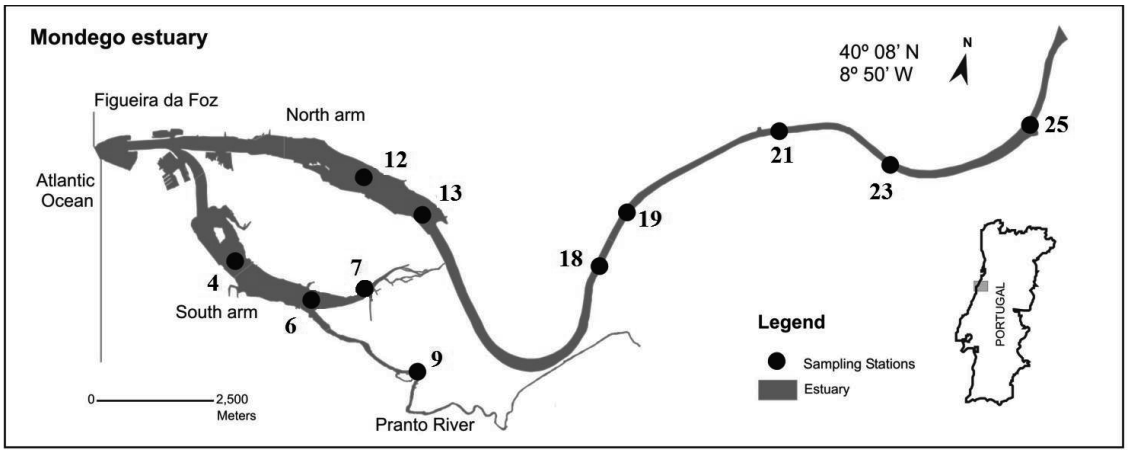


Figure 1. Mondego estuary (Portugal): station location (black circles). Areas: Euhaline (station 4), Polyhaline of the South Arm (stations 6, 7 and 9), Polyhaline of the North Arm (stations 12 and 13), Mesohaline (stations 18 and 19) and Oligohaline (stations 21, 23 and 25).

Environmental data

At each sampling station, bottom water parameters were measured *in situ* with a YSI Data Sonde Survey 4: salinity (Practical Salinity Scale) (in autumn 2009 - no salinity data was recorded), temperature (ºC), pH, and dissolved oxygen (DO) (mg L^{-1}). Water samples were collected for determination of nutrients and chlorophyll *a* (mg m^{-3}) in laboratory: nitrate (NO_3^--N) and nitrite (NO_2^--N) concentrations (µmol L^{-1}) were analysed according to standard methods described in Strickland and Parsons (1972) and ammonium (NH_4^+-N) and phosphate (PO_4^{3-}-P) concentrations (µmol L^{-1}) were analysed following the Limnologisk Metodik (1992). Chlorophyll *a* (Chl *a*) determinations were performed according to Parsons et al. (1985). Sediment samples were taken at each station to determine the organic matter content and grain size. Sediment organic matter (OM) content was defined as the difference between the weight of each sample after oven-drying at 60ºC for 72 h followed by combustion at 450ºC for 8 h, and was expressed as the percentage of the total weight. Grain size was analyzed by dry mechanical

separation through a column of sieves of different mesh sizes, corresponding to the five classes described by Brown and McLachlan (1990): a) gravel (>2 mm), b) coarse sand (0.500–2.000 mm), c) mean sand (0.250–0.500 mm), d) fine sand (0.063–0.250 mm), and e) silt and clay (<0.063 mm). The relative content of the different grain-size fractions was expressed as a percentage of the total sample weight.

Biological data

Three replicate samples of subtidal meiobenthos were collected, at each sampling station, by forcing a Kajak sediment corer (inner diameter: 4.6 cm) 3 cm into the sediment. All samples were preserved in 4% buffered formaldehyde and were sieved through 1 mm and 38 μm mesh size sieves (material retained on the smaller mesh was collected). Meiofauna was extracted from the sediment fraction using Ludox HS-40 colloidal silica at a specific gravity of 1.18 g cm^{-3} (Vincx, 1996). All meiobenthic organisms were identified to major taxa level under a stereomicroscope using Higgins and Thiel (1988) and Giere (2009) and the density (individuals per 10 cm^2) of each taxon was quantified.

From each replicate, a random set of 120 nematodes, or the total number of individuals in samples with less than 120 nematodes, were picked, cleared in glycerol–ethanol solution, transferred to anhydrous glycerol by evaporation and mounted on slides for identification (Vincx, 1996). All nematodes were identified to genus level using a microscope fitted with a x 100 oil immersion objective and based on the pictorial keys of Platt and Warwick (1983; 1988), Warwick et al. (1998), the online information system NeMys (Steyaert et al., 2005) and on Abebe et al. (2006).

Data analysis

Univariate and multivariate analyses to detect spatial and temporal changes in the community structure were performed according to the procedures described by Clarke (1993), using the PRIMER v6 software package (Clarke and Warwick, 2001) with the PERMANOVA add-on package (Anderson et al., 2008).

Environmental variables

A Principal Components Analysis (PCA) of the environmental variables was performed to find patterns in multi-dimensional data by reducing the number of dimensions, with minimal loss of information. Prior to the calculation of the environmental parameter resemblance matrix based on Euclidean distance, the environmental variables (temperature, salinity, dissolved oxygen, ammonium, nitrate, nitrite, phosphate, silicates, organic matter and each of the five granulometric classes) were square-root transformed (except dissolved oxygen and pH data) and followed normalisation.

Meiofauna assemblages

Total meiofauna density and density of individual major maiofauna taxa (individuals per 10 cm^2) were calculated, for each area and sampling occasion.

In order to test the hypothesis that the composition of meiofauna changes spatially and seasonally, a two–way PERMANOVA analysis was carried out with the following crossed factor design: "area" and "sampling occasion" as fixed factors, with five (Euhaline, Polyhaline North Arm, Polyhaline South Arm, Mesohaline and Oligohaline) and six levels (Su06, Au06, Wi07, Sp07, Su09 and Au09), respectively. Meiofauna taxa density data were square root transformed in order to scale down densities of highly abundant taxa and therefore increase the importance of the less abundant taxa in the analyses. The PERMANOVA test was conducted on Bray-Curtis similarity matrix and the residuals were permutated under a reduced model, with 9999 permutations. The null hypothesis was rejected when the significance level p was <0.05 (if the number of permutation was lower than 150, the Monte Carlo permutation p was used). If significant differences were detected, these were examined using *a posteriori* pair-wise comparisons, using 9999 permutations under a reduced model. Afterwards, the similarity between meiofauna assemblages along the estuary, in the different sampling occasions, was plotted using non-metric multidimensional scaling (nMDS), with Bray–Curtis as similarity measure (Clarke and Green, 1988).

Nematodes assemblages

As the Nematoda was always the dominant meiofaunal group, we decided to study this group in particular depth. Therefore, total density, genera diversity, trophic composition and several ecological indicators, either based on diversity (Margalef index, d; Shannon-Wiener diversity, H') or on ecological strategies (Index of Trophic Diversity, ITD; Maturity Index, MI), were calculated using the nematodes dataset, for each area and sampling occasion.

In order to investigate the trophic composition of the assemblages, marine nematodes genera were assigned to one of the four functional feeding groups, designated by Wieser (1953), based on buccal cavity morphology: selective (1A) and non-selective (1B) deposit feeders, epigrowth feeders (2A) and omnivores/predators (2B). The trophic classification of the freshwater nematodes was based on diet and buccal cavity structure information (Yeates et al., 1993; Traunspurger, 1997).

The Index of Trophic Diversity (Heip et al., 1985) was calculated as: $ITD = \sum \theta^2$, where θ is the density contribution of each trophic group to total nematode density, ranging from 0.25 (highest trophic diversity, i.e., each of the four trophic guilds account for 25% of the nematode density), to 1.0 (lowest trophic diversity, i.e., one trophic guild accounts for 100% of the nematode density). The Maturity Index (Bongers, 1990; Bongers et al., 1991) was used to analyze nematodes life strategy. Nematode genera were assigned a value on a scale (c-p score) accordingly their ability for colonizing or persisting in a certain habitat, from "colonizers" (c; organisms with a high tolerance to disturbance events) to "persisters" (p; low tolerance). Thus, the index is expressed as a c-p value, ranging from 1 (extreme colonizers) to 5 (extreme persisters) representing life-history characteristics associated with r- and *K*-selection, respectively (Bongers and Bongers, 1998; Bongers and Ferris, 1999) and varies from 1, under disturbed conditions, to 3 or 4, under undisturbed conditions. The index was calculated as the weighted average of the individual colonizer-persistent (c-p) values as

$M = \sum_{i=1}^{n} v(i) f.(i)$, where $v(i)$ is the c-p value of the taxon *i* and $f(i)$ is the frequency of that taxon.

Two-way permutational analyses of variance (PERMANOVA) were applied to test the null hypotheses that no significant spatial (between areas) and temporal (between sampling occasions) differences existed, in the nematode assemblage descriptors (total density, genera diversity, trophic composition, d, H', ITD and MI). PERMANOVA was used as an alternative to ANOVA since its assumptions were not met, even after data transformation. Two–way PERMANOVA analyses were carried out with the same design described for meiofauna analysis. All PERMANOVA tests were conducted on Euclidean-distance similarity matrices and the residuals were permutated under a reduced model, with 9999 permutations. The null hypothesis was rejected when the significance level *p* was <0.05 (if the number of permutation was lower than 150, the Monte Carlo permutation *p* was used). Whenever significant differences were detected, these were examined using *a posteriori* pair-wise comparisons, using 9999 permutations under a reduced model.

In order to test for temporal and spatial differences regarding nematodes assemblages' composition, a two–way PERMANOVA analysis was carried out with the previously described design ("area": 5 levels; "sampling occasion": 6 levels), using Bray-Curtis as similarity measure. The null hypothesis was rejected when the significance level *p* was <0.05 (if the number of permutation was lower than 150, the Monte Carlo permutation *p* was used). If significant differences were detected, these were examined using *a posteriori* pair-wise comparisons, using 9999 permutations under a reduced model. Nematode genera density data were first square root transformed in order to scale down densities of highly abundant genera and therefore increase the importance of the less abundant genera in the analyses, and the similarity between communities along the estuary, in the different sampling occasions, was plotted by non-metric multidimensional scaling (nMDS), using the Bray–Curtis similarity measure (Clarke and Green, 1988). Afterwards, the relative contribution of each genus to the average dissimilarities between areas and sampling occasions were calculated using two-way crossed similarity percentage analysis procedure (SIMPER, cut-off percentage: 90%).

Nematodes assemblages vs. environmental variables

The relationship between environmental variables and the structure of the nematodes community was explored by carrying out the BIOENV procedure (Clarke and Ainsworth, 1993), using Spearman's correlation.

RESULTS

Environmental variables

Along the estuary, salinity and nutrient concentrations showed opposite trends, with higher salinity values and lower nutrient concentrations downstream and lower salinity values and higher nutrient concentrations upstream. A decrease in grain size was also observed from Oligohaline area towards the mouth of the estuary.

The PCA ordination of the environmental factors showed that the first two components (PC1, 29.0% and PC2, 23.8%) accounted for about 53% of the variability of the data (Fig. 2). The Oligohaline and Mesohaline samples were characterized by high nutrients concentration, at all sampling occasions, while in autumn 2006, winter 2007 and spring 2007, the samples from these two upstream areas were clearly separated from the remaining ones mainly due to higher percentage of coarser sediments.

In general, independently from the sampling occasion, higher salinity, finer sediments and lower nutrient concentrations characterized the samples from the Polyhaline NA, Polyhaline SA and Euhaline areas. With a few exceptions (mainly in Summer 2009), the two Polyhaline areas presented different environmental attributes: the Polyhaline NA samples having coarser sediments and the Polyhaline SA samples being characterized by finer sediments and higher OM content.

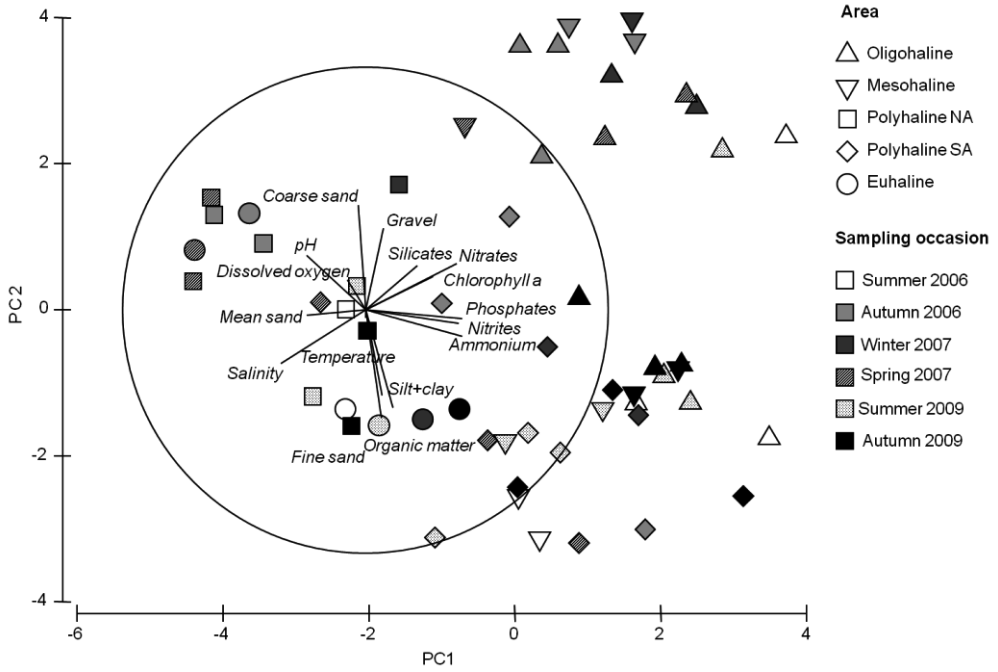


Figure 2. Principal component analysis (PCA) plot based on the environmental variables measured in each "area" (Oligohaline, Mesohaline, Polyhaline North Arm, Polyhaline South Arm and Euhaline) and "sampling occasion" (Summer 06, Autumn 06, Winter 07, Spring 07, Summer 09 and Autumn 09). PC1= 29.0%, PC2=23.8%.

Meiofauna assemblages

Fourteen major taxa were identified along the estuary during the sampling period with Nematoda the dominant taxon (92.4%), followed by Polychaeta (4.7%) and Harpacticoid copepods (1.5%). All other taxa attained less than 1% [e.g. Bivalvia (0.4%), Oligochaeta (0.4%), Ostracoda (0.2%), Tardigrada (0.1%), Gastropoda (0.1%), Amphipoda (0.1%), Nauplii (0.1%)] and some taxa presented very low density (less than 0.03%), such as Ciliophora, Halacaroidea, Turbellaria and Cladocera.

Total meiofauna density (± sd) ranged from 25.4 ± 25.9 ind.10cm^{-2} (Oligohaline, Sp07) to 1383.5 ± 687.9 ind.10cm^{-2} (Euhaline, Su06) and the number of taxa present varied from three (Mesohaline, Sp07; Euhaline, Au06 and Au09) to eleven (Polyhaline SA and Euhaline in Su06), with no clear increase from Oligohaline to Euhaline areas (Table 1). Permanova analysis of meiofauna

assemblage composition data showed a significant interaction between “area” and “sampling occasion” (Table 2A).

The Oligohaline area was different from all others on all sampling occasions, with minor exceptions in Au06 (Oligohaline similar to Euhaline, t=1.35, p=0.143), in Wi07 (Oligohaline only different from the Polyhaline SA, t=2.94, p=0.002) and in Sp07 (Oligohaline similar to Mesohaline, t=1.57, p=0.104). This pattern is distinctly visible in the nMDS ordination (Fig. 3), with a clear separation of Oligohaline and Mesohaline areas from the remaining ones.

Nematodes assemblages

Structure and trophic composition

The density (N) of nematodes ranged from 21.4 ± 23.5 ind $10cm^{-2}$ in the Oligohaline area (Sp07) to 1323.1 ± 674.7 ind $10cm^{-2}$ in the Euhaline area (Su06). Over the whole estuary, mean density (±sd) was highest in Wi07 (363.40±343.16 ind $10cm^{-2}$), and lowest during Au09 (123.04±154.79 ind $10cm^{-2}$). Generally, the highest densities were reached in the Euhaline and Polyhaline areas (Fig. 4A). Permanova analysis of density data showed a significant interaction between “area” and “sampling occasion” (Table 2B). Individual pair-wise comparisons on interaction factor (“area” x “sampling occasion”) showed that the Oligohaline area, in general, showed significantly lower density values than the other areas, regardless of the sampling occasion. Moreover, the Polyhaline NA did not show significant differences through time while all other areas showed significant differences in density between one or more sampling occasions (see Annex 1).

Table 1. Mean density ± standard deviation (number of individuals per 10 cm^2) of meiofaunal taxa in each area (Oligohaline, Mesohaline, Polyhaline North Arm, Polyhaline South Arm and Euhaline) and sampling occasion (summer 2006, Su06; autumn 2006, Au06; winter 2007, Wi07; spring 2007, Sp07; summer 2009, Su09 and autumn 2009, Au09).

Area	Sampling occasion	Nematoda	Polychaeta	Copepoda	Bivalvia	Oligochaeta	Ostracoda	Gastropoda	Nauplii	Tardigrada	Amphipoda	Ciliophora	Halacaroidea	Turbellaria	Cladocera	Total
Euhaline	**Su06**	1323.1±674.7	4.8±2.2	30.9±14.0	6.4±0.7	4.0±1.5	4.0±3.1	3.2±3.1	5.2±4.1		0.8±0.3			0.6±0.6	0.4±0.7	**1383.5±687.9**
	Au06	52.6±19.9	0.6±1.0	0.2±0.3												**53.4±20.5**
	Wi07	332.7±134.2	5.0±1.5	33.5±34.4		0.2±0.3	0.2±0.3	1.2±1.2	1.6±0.3							**374.5±160.2**
	Sp07	139.3±9.9	8.8±4.6	3.6±0.6		1.0±1.7										**152.7±10.5**
	Su09	157.5±63.4	0.6±1.0	2.8±2.4	1.0±1.3		0.2±0.3	1.2±1.2	0.2±0.3							**163.6±65.7**
	Au09	103.6±22.9		1.2±0.6				4.2±4.2								**109.0±26.2**
Polyhaline SA	**Su06**	617.0±468.7	42.3±22.8	14.2±18.3	0.6±0.5	0.4±0.5	5.9±8.4	0.1±0.1	0.1±0.1		0.1±0.2		1.1±1.0	0.1±0.1		**681.9±500.5**
	Au06	172.0±150.7	15.7±14.9	1.1±1.8			0.1±0.2									**189.0±161.8**
	Wi07	526.1±506.0	8.7±7.7	7.8±7.7	0.4±0.5	0.9±1.1		0.1±0.2	0.1±0.1							**544.0±521.8**
	Sp07	196.9±134.9	9.9±9.4	2.9±3.4	0.1±0.1	0.7±0.5							0.1±0.1			**210.6±145.0**
	Su09	201.2±81.0	7.8±1.5	2.4±2.3	0.3±0.3	0.1±0.1	1.1±0.9		0.1±0.1							**212.9±77.5**
	Au09	182.6±70.7	9.5±4.5	1.5±1.3	0.1±0.2		0.2±0.2	0.1±0.1			0.1±0.1	0.1±0.1				**194.3±69.2**
Polyhaline NA	**Su06**	238.4±13.6	16.8±10.4	4.0±4.0	1.2±0.6	3.2±2.8	0.1±0.1	1.0±1.4	0.4±0.3			1.8±3.6	0.1±0.1			**267.0±1.3**
	Au06	259.9±14.8	2.3±1.3		0.1±0.1			0.1±0.1								**262.4±16.0**
	Wi07	72.8±103.0	1.7±2.4	0.3±0.3		0.4±0.6		0.1±0.1	0.1±0.1							**75.5±106.7**
	Sp07	173.5±98.3	10.1±9.5	0.2±0.3		0.1±0.1			0.1±0.1							**184.0±108.1**
	Su09	303.7±115.9	2.4±0.0	1.1±1.3	0.1±0.1	5.7±7.5		0.1±0.1								**313.2±121.9**
	Au09	247.0±100.2	2.4±2.8	0.7±1.0	0.2±0.3	1.9±1.8										**252.3±97.9**
Mesohaline	**Su06**	183.8±1.7	63.8±24.4	2.2±2.6	0.5±0.4	0.5±0.7	1.2±0.3				0.2±0.0	0.3±0.4	0.1±0.1	0.2±0.3		**252.9±28.7**
	Au06	260.5±16.5	2.0±0.6			0.1±0.1	0.1±0.1				0.2±0.3		0.1±0.1			**263.0±15.2**
	Wi07	209.8±106.3	5.4±0.3	0.2±0.3	0.2±0.3	0.1±0.1	0.5±0.7									**216.2±105.7**
	Sp07	68.0±74.6	2.4±1.1	0.2±0.3												**70.6±75.5**
	Su09	55.6±43.7	6.0±1.7	0.5±0.1	0.3±0.4	1.6±2.3										**64.0±44.6**
	Au09	55.1±17.5	1.0±0.0	0.6±0.9		0.5±0.4					1.1±1.0					**58.3±17.2**
Oligohaline	**Su06**	85.8±41.4	29.2±11.7	1.5±1.3	12.3±18.8	0.5±0.8	0.1±0.1		0.2±0.2		0.2±0.2					**129.7±43.5**
	Au06	23.9±6.0	0.7±0.6	0.1±0.1		1.9±3.2					0.1±0.1		0.1±0.1			**26.8±7.5**
	Wi07	67.4±82.0	4.5±6.9	0.3±0.4	0.3±0.6	0.1±0.1			0.1±0.1	5.4±9.4						**78.2±98.5**
	Sp07	21.4±23.5	1.9±2.3	1.6±0.9		0.2±0.3	0.2±0.2			0.1±0.1						**25.4±25.9**
	Su09	29.7±22.8	1.5±1.0	3.0±4.5	2.2±3.8	0.1±0.2										**36.6±19.5**
	Au09	32.6±17.3	1.2±0.2	0.7±0.5	0.1±0.1	0.1±0.1	0.1±0.2	0.1±0.1			2.5±3.6				0.2±0.2	**37.5±21.2**

Table 2. Details of the two-factor Permanova test ("area" with 5 levels, and "sampling occasion" with 6 levels, as fixed factors) for all variables analyzed. Bold values stand for the significant differences (p<0.05). A – Meiofauna composition; B – Nematodes descriptors.

	Source of variation	Degrees of freedom	Sum of squares	Mean squares	Pseudo-F	P(perm)
A. Meiofauna						
Composition	Area	4	39752	9937.9	16.28	**0.0001**
	Sampling occasion	5	23716	4743.3	7.77	**0.0001**
	Area x Sampling occasion	19	24391	1283.7	2.10	**0.0001**
	Residual	139	84871	610.58		
	Total	167	175020			
B. Nematodes						
Total density	Area	4	2423900	605970	24.31	**0.0001**
	Sampling occasion	5	2012300	404860	16.24	**0.0001**
	Area x Sampling occasion	19	4162200	219060	8.79	**0.0001**
	Residual	139	3464500	24925		
	Total	167	10996000			
Number of genera	Area	4	471.19	117.8	10.37	**0.0001**
	Sampling occasion	5	318.13	63.626	5.60	**0.0001**
	Area x Sampling occasion	19	373.84	19.676	1.73	**0.0401**
	Residual	139	1578.6	11.357		
	Total	167	2823.6			
Trophic composition	Area	4	19645	4911.3	8.10	**0.0001**
	Sampling occasion	5	19402	3880.4	6.40	**0.0001**
	Area x Sampling occasion	19	22170	1166.9	1.92	**0.0006**
	Residual	139	84261	606.2		
	Total	167	150940			
Composition	Area	4	98388	24597	16.37	**0.0001**
	Sampling occasion	5	37623	7524.6	5.01	**0.0001**
	Area x Sampling occasion	19	61000	3210.5	2.14	**0.0001**
	Residual	139	208840	1502.4		
	Total	167	420420			
Margalef Index	Area	4	48.505	12.126	21.99	**0.0001**
	Sampling occasion	5	4.5976	0.91952	1.67	0.152
	Area x Sampling occasion	19	19.238	1.0125	1.84	**0.025**
	Residual	139	76.665	0.55154		
	Total	167	155.88			
Shannon-Wiener	Area	4	13.633	3.4082	8.22	**0.0001**
	Sampling occasion	5	2.0816	0.41632	1.00	0.4157
	Area x Sampling occasion	19	11.831	0.62267	1.50	0.0972
	Residual	139	57.633	0.41462		
	Total	167	87.925			
Index of Trophic	Area	4	0.31339	0.078347	3.05	**0.0203**
	Sampling occasion	5	0.11341	0.022682	0.88	0.4951
	Area x Sampling occasion	19	0.59974	0.031565	1.23	0.2383
	Residual	139	3.5658	0.025653		
	Total	167	4.5852			
Maturity Index	Area	4	4.1698	1.0425	9.86	**0.0001**
	Sampling occasion	5	0.99525	0.19905	1.88	0.1054
	Area x Sampling occasion	19	3.5231	0.18543	1.75	**0.0438**
	Residual	139	14.701	0.10576		
	Total	167	24.568			

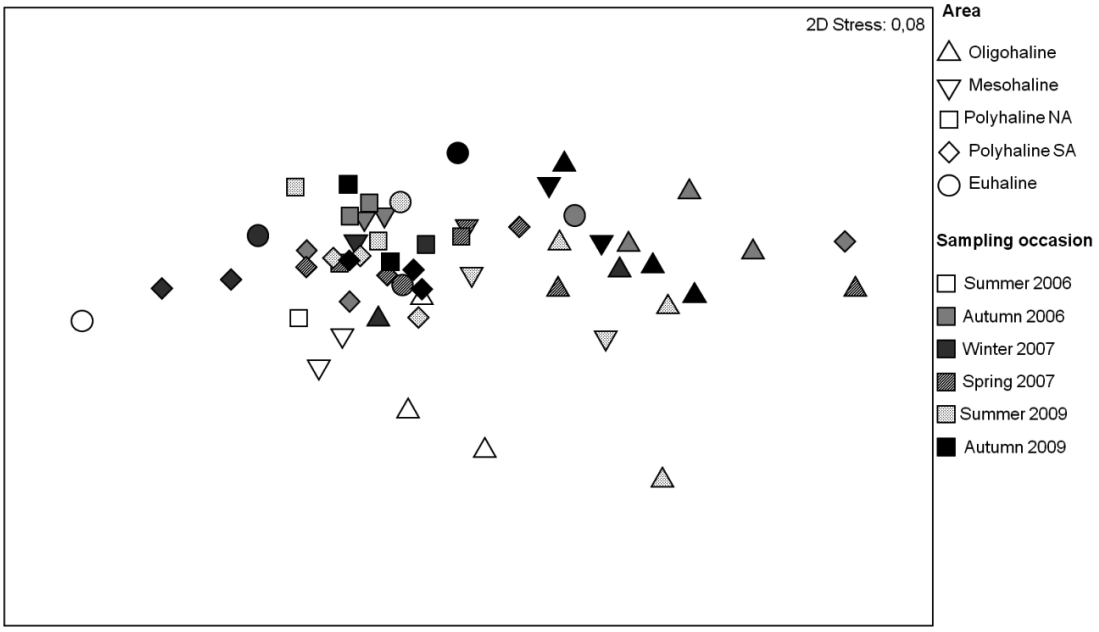
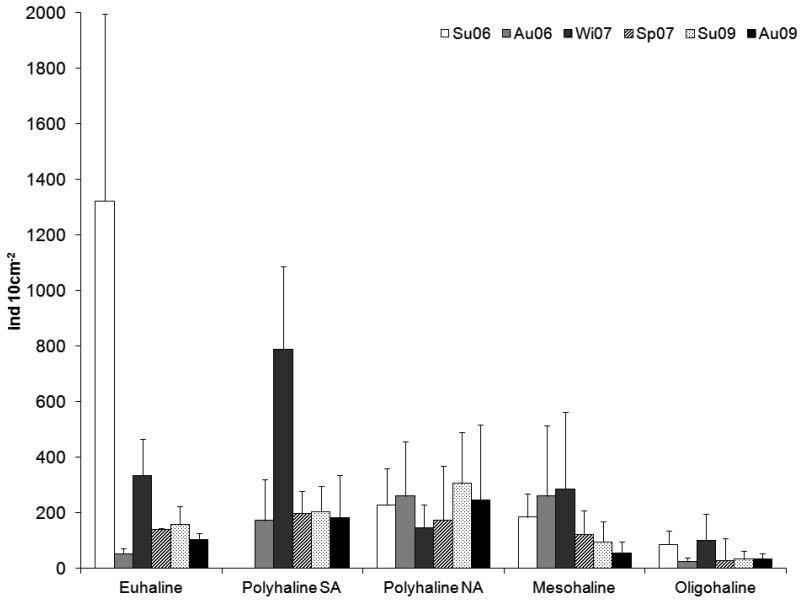


Figure 3. nMDS ordination based on meiobenthos in each of the sampling stations in each "area" (Oligohaline, Mesohaline, Polyhaline North Arm, Polyhaline South Arm and Euhaline) and "sampling occasion" (Summer 06, Autumn 06, Winter 07, Spring 07, Summer 09 and Autumn 09).

Nematodes accounted for between 88% (Su06) to 95% (Au06) of the total meiofaunal density and a total of 106 nematode genera, belonging to 40 families, were identified along the estuary during the study period. The most abundant orders were Chromadorida (46.3%), Monhysterida (36.7%) and Enoplida (11.7%) and the most abundant families were Comesomatidae (25.3%), Xyalidae (16.7%), Linhomoeidae (11.8%), Chromadoridae (10.3%) and Sphaerolaimidae (8.6%).

The number of genera (S) ranged between 8 in the Polyhaline NA area (Su09) and 19 in the Euhaline area (Su06) (Fig. 4B). Permanova revealed a significant interaction of factors "area" and "sampling occasion" for the number of genera (Table 2B). The pair-wise tests performed on the interaction term showed that in Au06, Sp07 and Au09 there were no significant differences in number of genera between areas, while in the remaining sampling occasions the Euhaline area showed higher diversity than the other areas. All areas showed significant variation in the number of genera between at least two sampling occasions (see Annex 1).

A. Average density



B. Number of genera

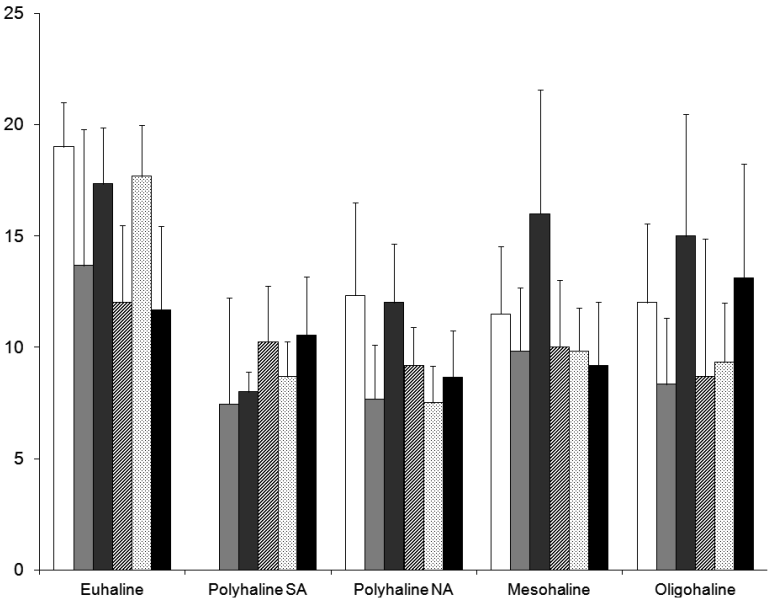


Figure 4. Nematode community in each "area" (Oligohaline, Mesohaline, Polyhaline North Arm, Polyhaline South Arm and Euhaline) during the study period (Su06,summer 2006; Au06, autumn 2006; Wi07, winter 2007; Sp07, spring 2007; Su09, summer 2009; Au09, autumn 2009). A) Average density (ind 10 cm^{-2}); B) Number of genera (S).

Throughout the study period, fifteen genera dominated the nematode assemblages (90.8%): *Sabatieria*, *Daptonema*, *Terschellingia*, *Metachromadora*, *Sphaerolaimus*, *Anoplostoma*, *Dichromadora*, *Viscosia*, *Ptycholaimellus*, *Microlaimus*,

Linhomoeus, *Axonolaimus*, *Paracyatholaimus*, *Mesodorylaimus* and *Prochromadorella* (Table 3). The remaining genera all represented abundances lower than 1%. The most spatially widespread genus was *Daptonema* (present along the whole length of the estuary through the entire sampling period), followed by *Sabatieria* and *Dichromadora* (Table 3). Freshwater nematodes comprised 3.5% of the total nematodes density (1% in Sp07 to 4.4% in Wi07).

The five dominant genera showed clear variation over the study period, as shown in Fig. 5, and a distinct pattern of genera turnover along the estuary is visible. Non-selective deposit feeders (1B) like *Sabatieria* and *Daptonema*, showed an opposite density contribution trend in the Polyhaline areas, with the contribution of *Sabatieria* increasing from Wi07 to Au09, and *Daptonema* decreasing in the same period. *Sabatieria* was almost absent in the Mesohaline and Oligohaline areas, where *Daptonema* showed a high contribution. *Terschellingia*, a selective deposit feeder (1A), showed high contributions in Wi07, especially in the Polyhaline SA and Mesohaline areas. Predators (2B), like *Metachromadora* and *Sphaerolaimus*, peaked on different sampling occasions, with a high contribution of *Metachromadora* in the Euhaline area, while *Sphaerolaimus* was mostly observed in the Polyhaline NA (Au06) and Mesohaline (Wi07) areas.

Throughout the estuary, the nematodes community was characterized by a dominance of non-selective deposit feeders (52.0±12.1%) during the entire study period, followed by omnivores/predators (23.2±8.1%), epigrowth feeders (15.9± 3.3%) and selective deposit feeders (8.9±4.8%). Non-selective deposit feeders were the most abundant trophic group, in almost all areas and sampling occasions, ranging from 22.5% (Euhaline area, Au06) to 81.6% (Polyhaline NA area, Au09). In the Mesohaline and Oligohaline areas there was a lower contribution of predators on all sampling occasions (ranging from 1.7% in Au06 to 16.6% in Wi07, both in the Mesohaline area) compared with the remaining areas (ranging from 7.3% in Au09, Polyhaline NA area to 56.7% in Au06, Euhaline area) (Fig. 6). Permanova analysis of trophic structure data showed a significant interaction between factor "area" and "sampling occasion" (Table 2B). Individual pair-wise comparisons performed on the interaction factor showed significant differences in trophic

composition between areas on all sampling occasions and also significant differences at each area throughout the study period (Annex 1).

Regarding the overall composition, multivariate Permanova analysis showed that the estuarine assemblages were different between areas and sampling occasions (Table 2B). In concrete, depending on the chosen area, there were significant differences between distinct pair of sampling occasions. The results are supported by a visual assessment of the patterns in the nMDS ordination of square-root transformed data, using Bray-Curtis, as shown in Fig. 7.

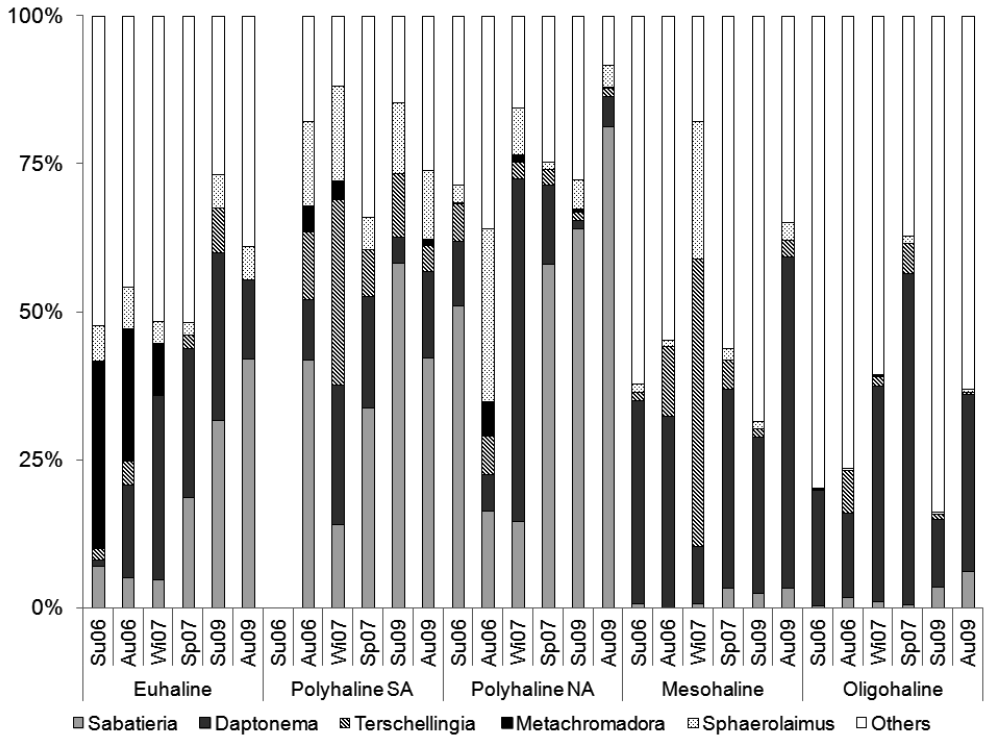


Figure 5. Percentage of contribution of the five most abundant nematode genera (*Sabatieria*, *Daptonema*, *Terschellingia*, *Metachromadora* and *Sphaerolaimus*) in each "area" (Oligohaline, Mesohaline, Polyhaline North Arm, Polyhaline South Arm and Euhaline) and "sampling occasion" (Summer 06, Autumn 06, Winter 07, Spring 07, Summer 09 and Autumn 09).

Two-way SIMPER analysis showed how the nematodes genera contributed to similarity values of the *a priori* defined groups. Maximum dissimilarities were obtained between the Oligohaline area and both the Polyhaline areas (80.15% with Polyhaline SA and 79.57% with Polyhaline NA) and Euhaline area (79.78%). Maximum dissimilarities were also observed between Summer 06 and the

following three sampling occasions, Autumn 06 (71.57%), Winter 07 (68.59%) and Spring 07 (68.58%). The genera that contributed most to the similarity within both sampling occasions and areas were *Daptonema*, *Sabatieria*, *Sphaerolaimus* and *Dichromadora*.

Indices estimation

Margalef index (d) and Shannon-Wiener index values (H') (Fig. 8A), followed the trend shown by the number of genera (Spearman correlation = 0.74 and 0.72, respectively; $p<0.05$). The Margalef index showed a significant interaction between "area" and "sampling occasion" (Table 2B). The Mesohaline and Euhaline areas did not show significant differences in richness throughout the study period, while the Oligohaline area showed several pairs of sampling occasions with significantly different richness values, higher in Wi07 and Au09. Moreover, no significant differences where found between areas in Su06 and Sp07 (Annex 1). The Shannon-Wiener index showed significant differences between all pairs of areas (Table 2B) except between Oligohaline - Mesohaline ($t=1.27$, $p=0.21$) and Mesohaline -Polyhaline SA ($t=1.24$; $p=0.22$). In general, both indicators showed a lower diversity in the Polyhaline areas (Fig. 8A).

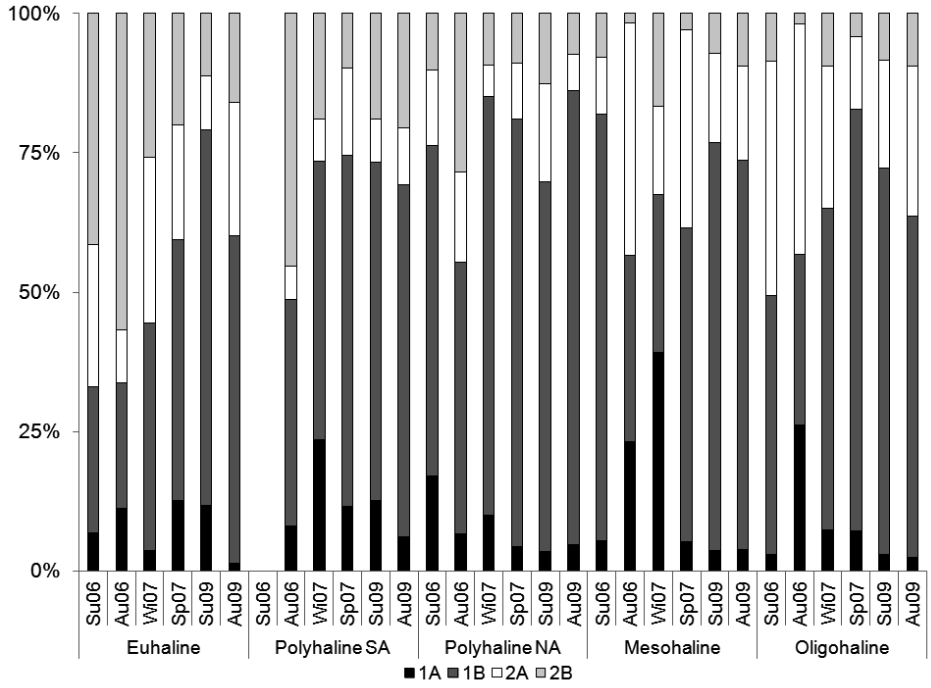


Figure 6. Percentage of contribution of the different trophic groups, in each "area" (Oligohaline, Mesohaline, Polyhaline North Arm, Polyhaline South Arm and Euhaline) and "sampling occasion" (Summer 06, Autumn 06, Winter 07, Spring 07, Summer 09 and Autumn 09). 1A – selective deposit feeders; 1B – non-selective deposit feeders; 2A – epigrowth feeders; 2B – omnivores/predators.

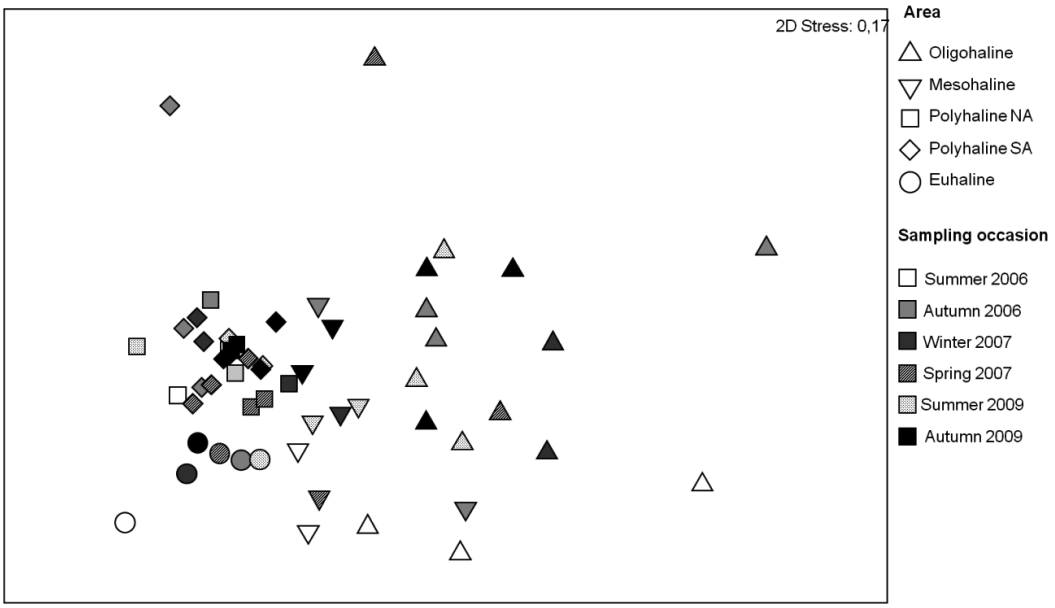
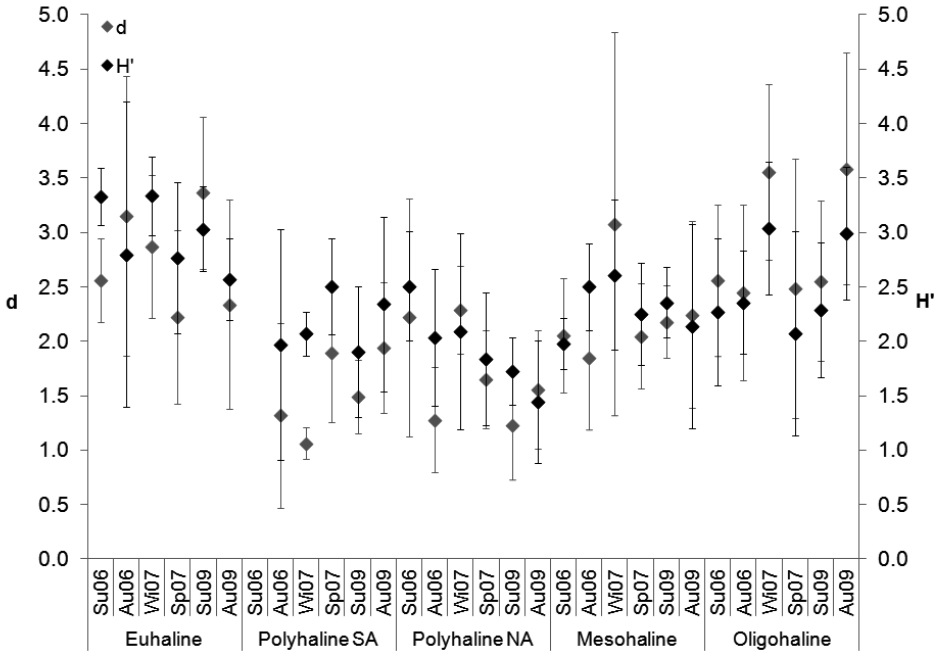


Figure 7. nMDS ordination based on nematodes dataset in each "area" (Oligohaline, Mesohaline, Polyhaline North Arm, Polyhaline South Arm and Euhaline) and "sampling occasion" (Summer 06, Autumn 06, Winter 07, Spring 07, Summer 09 and Autumn 09).

The Index of Trophic Diversity ranged from 0.31 (Euhaline, Su06) to 0.62 (Polyhaline NA, Sp07). Significant differences were observed between areas (Table 2B), with higher values in the Oligohaline and Mesohaline areas, indicating lower trophic diversity, and lower values in the Polyhaline and Euhaline areas (Polyhaline NA>Polyhaline SA, Polyhaline NA>Euhaline), indicative of a higher trophic diversity (Fig. 8B).

The Maturity Index (MI) ranged between 2.1 (Polyhaline NA in Wi07, Sp07, Su09 and Au09; Mesohaline in Su06 and Sp07) and 3.0 (Oligohaline, Su06) (Fig.8B) and most nematodes showed a c-p value of 2 (average=70%), followed by c-p values of 3 (26%). The MI showed a significant interaction between the factors “area” and “sampling occasion” (Table 2B). Individual pair-wise comparisons performed on the interaction revealed no seasonal differences in the Polyhaline SA area. The MI values of the Mesohaline area exhibited the highest temporal variations. Interestingly, in Au06 (flood period), no significant differences in MI were recorded along the estuary.

A. Margalef (d) and Shannon-Wiener (H') indices



B. Maturity Index (MI) and Index of Trophic Diversity (ITD)

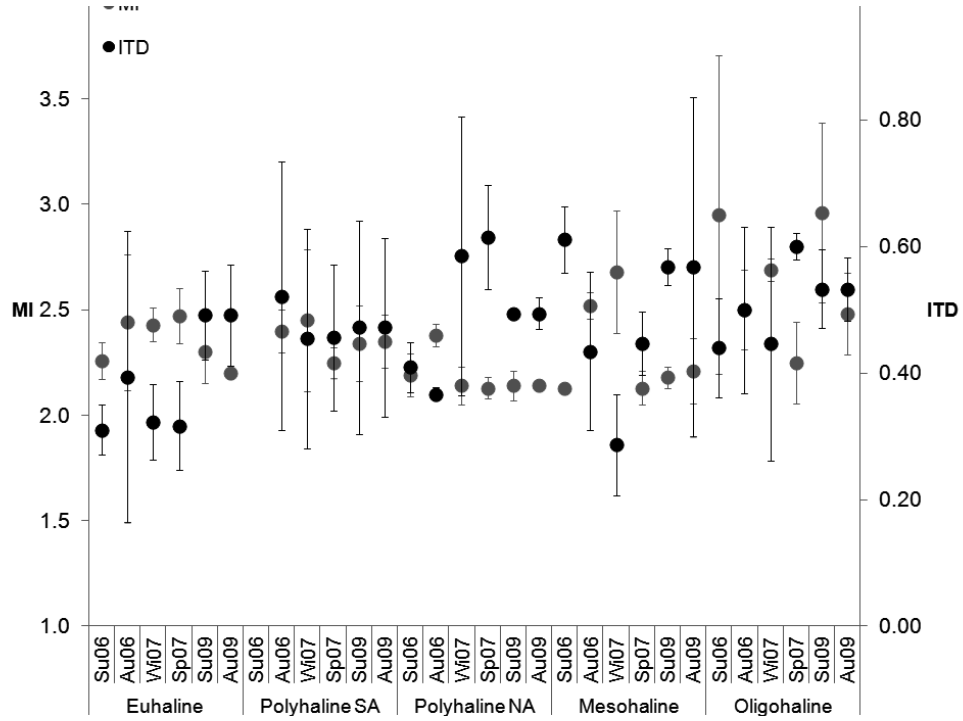


Figure 8. Ecological indicators values in each "area" (Oligohaline, Mesohaline, Polyhaline North Arm, Polyhaline South Arm and Euhaline) and "sampling occasion" (Summer 06, Autumn 06, Winter 07, Spring 07, Summer 09 and Autumn 09). A) Margalef index (d ± standard deviation) and Shannon- Wiener index (H' ± standard deviation) (bits ind^{-1}); B) Index of Trophic Diversity (ITD ± standard deviation) and Maturity index (MI ± standard deviation).

Table 3. Average density ($\bar{x}$; number individuals per 10cm^2), percentage of contribution (%) and rank by density (Rk) of nematode genera in each area of the Mondego estuary derived from pooled data from all sampling occasions. Table only lists the genera that contributed >0.5% to the total density and the five most abundant genera in each area are shaded.

			Euhaline			Polyhaline SA			Polyhaline NA			Mesohaline			Oligohaline		
Genera	**Total average density**	**%**	$\bar{x}$	%	**Rk**	$\bar{x}$	**%**	**Rk**	$\bar{x}$	**%**	**Rk**	$\bar{x}$	**%**	**Rk**	$\bar{x}$	**%**	**Rk**
Sabatieria	249.9	23.5	38.5	10.9	2	87.5	31.9	1	121.7	51.9	1	1.6	1.0	12	0.5	1.1	16
Daptonema	163.1	15.3	36.4	10.3	3	45.9	16.7	3	22.2	9.5	3	47.6	29.8	1	11.1	25.8	1
Terschellingia	86.7	8.2	7.3	2.1	13	49.3	18.0	2	7.8	3.3	7	21.6	13.5	3	0.8	1.8	12
Metachromadora	86.0	8.1	76.7	21.8	1	5.6	2.0	9	3.6	1.5	10	0.1	0.0	45	0.1	0.1	47
Sphaerolaimus	84.6	8.0	18.9	5.4	6	34.9	12.7	4	22.2	9.5	2	8.5	5.3	6	0.1	0.3	30
Anoplostoma	75.5	7.1	25.3	7.2	5	9.5	3.5	6	10.2	4.3	5	28.2	17.7	2	2.2	5.2	4
Dichromadora	47.1	4.4	5.0	1.4	18	5.5	2.0	10	20.9	8.9	4	13.6	8.5	4	2.2	5.1	5
Viscosia	37.0	3.5	14.4	4.1	7	8.9	3.3	7	9.1	3.9	6	4.3	2.7	8	0.3	0.7	19
Ptycholaimellus	35.4	3.3	8.4	2.4	11	10.5	3.8	5	4.8	2.1	8	10.4	6.5	5	1.3	3.1	7
Microlaimus	30.1	2.8	29.7	8.4	4				0.4	0.2	16				0.0	0.1	56
Linhomoeus	18.5	1.7	8.8	2.5	10	7.7	2.8	8	1.8	0.8	11	0.1	0.0	38	0.1	0.3	28
Axonolaimus	14.4	1.4	11.1	3.2	8	0.1	0.0	26	0.5	0.2	14	1.6	1.0	11	1.0	2.4	9
Paracyatholaimus	13.4	1.3	2.4	0.7	22	0.1	0.0	28	0.3	0.1	17	7.4	4.6	7	3.3	7.6	3
Mesodorylaimus	12.5	1.2	0.4	0.1	35							2.8	1.7	10	9.4	21.8	2
Prochromadorella	11.0	1.0	10.7	3.0	9	0.1	0.0	27	0.1	0.1	23						
Leptolaimus	8.4	0.8	1.5	0.4	27	0.6	0.2	16	4.5	1.9	9	1.4	0.9	13	0.4	0.9	18
Molgolaimus	8.0	0.8	7.7	2.2	12	0.2	0.1	25	0.1	0.0	28	0.1	0.0	40			
Calyptronema	7.0	0.7	6.4	1.8	14	0.7	0.2	15									
Chromadora	6.4	0.6	5.2	1.5	16	0.6	0.2	17	0.5	0.2	13						
Spilophorella	5.6	0.5				0.2	0.1	23	1.7	0.7	12	3.5	2.2	9	0.1	0.3	29
Aegialoalaimus	5.5	0.5	5.5	1.6	15												
Halalaimus	5.4	0.5	3.9	1.1	20	0.9	0.3	13	0.3	0.1	18	0.2	0.1	26	0.2	0.4	26
Paralinhomoeus	5.2	0.5	5.1	1.4	17							0.1	0.0	41			
Oncholaimellus	5.0	0.5	4.7	1.3	19							0.2	0.1	29	0.1	0.1	40
Other genera	41.3	3.9	17.8	5.1		5.3	1.9		1.9	0.8		6.5	4.1		9.8	22.8	
Mean density			351.7			274.0			234.8			159.6			43.0		
Total genera			53			33			35			58			84		

Environmental variables vs. nematode assemblages

Separate BIOENV analysis were performed for each sampling occasion in order to analyze the main factors responsible for the distribution of nematodes along the estuary in each sampling occasion, with salinity, grain size variables and nutrients always being correlated with the nematode assemblage composition (Table 4).

Table 4. BIOENV results carried out for nematodes assemblages and environmental data, in each sampling occasion.

Sampling occasion	Spearman's rank correlation	Variables
Summer 2006	0.938	Salinity,NO_3^-, mean sand , coarse sand, Chl *a*
Autumn 2006	0.245	pH, fine sand, coarse sand
Winter 2007	0.636	Salinity, pH, mean sand
Spring 2007	0.839	Salinity, NO_3^-
Summer 2009	0.862	Salinity, NO_3^-
Autumn 2009	0.642	NO_3^-, silicates. %OM, mean sand

DISCUSSION

The combination of the temporal and spatial information on meiofauna and nematodes of the Mondego estuary allowed a full description of the meiobenthic communities along the estuarine gradient to be made. The information was then analyzed in the context of the ecological assessment of transitional waters using these communities, making available information on the ecological conditions of the system and initiating a baseline for long-term monitoring studies. Previous studies have only been focused on one season, lacking temporal replication (Alves et al., 2009; Adão et al., 2009; Patrício et al., 2012), and the present study, as well as integrating the complete estuarine gradient, was repeated on six sampling occasions, allowing a more extensive database to be analyzed and related to the environmental gradient.

The environmental characterization of the Mondego estuary was based on abiotic measurements collected at each sampling event. The characterization of a system based on chemical parameters only provides information about quality at the time of measurement, lacking the sensitivity to determine the impact of

previous events on the ecology of the system (Spellman and Drinan, 2001). However, bioindicators provide indications about past conditions and to accurately assess ecological conditions it is necessary to use a set of indicators which represent the structure, function and composition of the system. In this study, meiobenthic communities were studied in detail, with special emphasis on nematodes assemblages.

A clear estuarine gradient, from the oligohaline area toward the euhaline zone was observed during the survey period, mainly caused by variations in salinity, nutrient concentrations and sediment grain size. The identification of both arms of the Mondego estuary as two different subsystems was confirmed, representing distinct hydrological regimes. Salinity increased from upstream towards the mouth of the estuary on all sampling occasions except in autumn 2006. During this season, a period of heavy rain and flooding occurred (INAG source), lowering salinity values and confirming the importance of extreme events in changing the environmental characteristics of estuaries. The nematode community was affected at this time since the separation of salinity zones along the estuary was not so distinct. The severe flood may have caused sediment displacement and erosion as well as changing the interstitial water salinity (Santos et al., 1996), and organisms may have been washed away, leading to the low density values observed during this season.

Both salinity and sediment structure are major factors influencing meiobenthic community structure (Heip et al., 1985) and results from the BIOENV analysis showed that the distribution pattern of nematodes was mainly structured by distinct environmental factors like salinity, sediment grain size and water nutrients, supporting the primary influence of the estuarine gradient on nematode community patterns (Austen and Warwick, 1989; Vincx et al., 1990; Coull, 1999; Ferrero et al., 2008; Schratzberger et al., 2008; Adão et al., 2009). However, despite the other environmental differences between the polyhaline areas, the meiofauna and nematode communities were similar, emphasizing the prime importance of salinity in defining and limiting species distribution in transitional water systems (Austen and Warwick, 1989; Vincx et al., 1990; Soetaert et al., 1995; Attrill, 2002; Ferrero et al., 2008), its effects overriding that of sediment grain size composition (Austen and Warwick, 1989; Adão et al., 2009).

Meiofauna density and diversity were similar to other meiofauna communities, with densities falling within the range observed in other European estuaries (Smol et al., 1994; Soetaert et al., 1994; 1995). The dominance of nematodes over all other taxa is well documented, with Nematoda typically being the most abundant taxon (usually 60-90%) (Coull, 1999). Polychaeta ranked second, contrary to the common observation that copepods are usually more abundant (Coull, 1999). Harpacticoid copepods are sensitive to environmental perturbation (Hicks and Coull, 1983; Van Damme et al., 1984) and the low densities observed may indicate anthropogenic disturbances in the Mondego estuary. Low density of harpacticoid copepods was also observed in the Westerschelde (Van Damme et al., 1984; Soetaert et al., 1995) and was ascribed to pollution effects.

The increase in taxonomic resolution (from meiofauna major taxa to nematode genus level) enhanced our knowledge of the system, suggesting that higher taxonomic resolution may be more informative for measurement of changes in meiofauna community structure. However, some studies of meiofauna communities as indicators of status in marine environments (Schratzberger et al., 2000) and as indicators of pollution in harbours (Moreno et al., 2008), for instance, have shown that meiofauna taxon assemblages could provide a sensitive and clear measure of environmental status when comparing inshore and offshore locations and that indicators based on meiofauna taxa demonstrated a significant correlation with the concentration of contaminants.

Nematodes communities comprised a high number of genera but with few dominant ones, as observed in other estuaries (Austen et al., 1989; Li and Vincx, 1993; Soetaert et al., 1995; Rzeznik-Orignac et al., 2003; Steyaert et al., 2003; Ferrero et al., 2008). The dominant genera were similar to those found in the Brouage mudflat (France) (Rzeznik-Orignac et al., 2003) and in the Thames estuary (United Kingdom) (Ferrero at al., 2008), indicating that species that are able to tolerate the highly variable salinity in estuaries tend to be abundant, taking advantage of the plentiful food resources of estuaries (Hourston et al., 2011). Also, the wide distribution range of *Daptonema*, *Sabatieria* and *Dichromadora*, also observed by Ferrero et al. (2008), reflects the wide salinity range tolerated by these genera (Heip et al., 1985; Moens and Vincx, 2000; Ferrero et al., 2008).

Moreover, *Sabatieria*, *Daptonema* and *Terschellingia,* the three most abundant genera in the present study, are known to be tolerant to pollution (Soetaert et al., 1995; Austen and Somerfield, 1997; Schratzberger et al., 2006; Steyaert et al., 2007; Armenteros et al., 2009; Gambi et al., 2009), and their high densities along the Mondego estuary may be indicative of the pressures from which this estuary suffers. In fact, Moreno et al. (2011), in an evaluation of the use of nematodes as biological indicators of environmental quality in sediments of the Mediterranean Sea stated that the presence of some genera provided accurate information on the ecology and adaptation of organisms to environmental conditions. In this study, disturbed places were characterized by a high density of *Terschellingia*, *Paracomesoma* and *Sabatieira*, and sites classified as in moderate or poor ecological quality status were also dominated by *Daptonema*, indicating that such inhospitable habitat conditions can only be tolerated by genera able to thrive in extreme conditions (Moreno et al., 2008).

Genera diversity broadly followed the Remane's diagram (1934) for the effect of the salinity gradient on benthic invertebrates species richness (postulated for the Baltic Sea), with high diversity in the more stable marine and freshwater waters. According to Attrill (2002), salinity variation over time may be more important than average salinity for the distribution of nematodes along the estuary (also confirmed by Ferrero et al., 2008). The premise that environmental variables influence meiobenthic communities is well described, but the question of how far back we should consider the environmental history of a system in order to explain the distribution of the communities depends on the life-history characteristics of the species and, coupled with the characterization of the environment, extreme events should also be taken in consideration (Soetaert et al., 1995).

Spatial variability, with the transition between areas being characterized by different assemblages and with strong variations in genera dominance, was detected. The shift from an oligohaline nematode community, characterized by low density, high nematode diversity and high abundance of *Daptonema*, to a typical estuarine community, characterized by high nematode density, was observed, as in the Thames estuary (Ferrero et al., 2008). The remaining areas were also discrete, each one characterized by a different community, with the exception of the Polyhaline areas (see above).

In the present study, besides the clear spatial pattern, some temporal variations were also observed. Similar results were observed in the Swan River estuary, Australia (Hourston et al., 2009), with nematode species being markedly influenced by both site and season, with site being the most important factor. In temperate regions, nematode densities usually peak in the warmest months (Hicks and Coull, 1983; Smol et al., 1994) and in this study, although the highest density was observed in summer 2006, the pattern was not repeated in the other warm seasons.

The multivariate analysis allowed a representation of both environmental and biological (meiofauna and nematodes) data, showing that the estuarine abiotic gradient was mostly reflected in the biological communities.

Spatial and temporal variations of nematode assemblages has been studied in several systems (e.g. Yodnarasri et al., 2008; Armenteros et al., 2009; Hourston et al., 2009; Semprucci et al., 2010; Hourston et al., 2011) and, in order to use that information for ecological assessment, the application of ecological indices to the nematodes assemblages enhanced our knowledge on the benthic environment. Coupled with the taxonomic diversity, functional diversity is important for interpreting distribution patterns of the communities (Schratzberger et al., 2008). In what refers to meiobenthic communities, and besides the common diversity measures, specific indicators rely on nematodes information, such as the Maturity Index and the Index of Trophic Diversity. These two indices do not depend on the system, not suffering from lack of generality and the use of indicators based on different ecological principles is, according to Dauer et al., (1993) highly recommended in determining the environmental quality status of an ecosystem (Marques et al., 2009).

Knowing that the Mondego estuary suffers from anthropogenic pressures, especially in the Polyhaline areas (Northern arm - dredging activities, harbour; Southern arm – inputs from the Pranto River and agricultural runoffs), we can evaluate the performance of the indices in differentiating homogeneous sectors of impact along the estuary. The results verified that the indices behaved differently. For example, the Index of Trophic Diversity, generally used to correlate trophic diversity with pollution levels (Heip et al., 1985), appeared only to differentiate “extreme” conditions such as the relatively good ecological conditions in the mouth

of the estuary (reflected in high trophic diversity index values) and the upstream part of the estuary having lower ecological status. In the upstream zone, the incorporation of feeding information on the freshwater genera, mostly predators, may have contributed to the observed pattern. However, if this dominance is a natural feature in estuaries, the parameters of this index should be readjusted so that the predominance of freshwater nematodes does not exclusively imply a classification of bad ecological conditions. A similar result was observed by Moreno et al. (2011), with the ITD not separating sites with different ecological classifications and even indicating a good Ecological Quality Status in disturbed sites.

Furthermore, the classification of feeding complexity, as first described by Wieser (1953), has the disadvantage of confining nematode species to a single trophic status (Heip et al., 1985), which may not represent the real complexity of feeding habitats of nematodes (Moens and Vincx, 1997), with trophic plasticity being described for most feeding types (Moens et al., 2005; Schratzberger et al., 2008). On the other hand, the low Maturity index values observed in both the polyhaline and euhaline areas suggested a high stress level, since opportunistic genera increase in abundance in adverse conditions (Bongers and Bongers, 1998; Gyedu-Ababio and Baird, 2006). An opposite trend was observed in the oligohaline area, where the MI reached maximum values, indicating a better ecological status, with the MI also capturing the composition variations that occurred in the upstream area over time (higher dispersion of oligohaline samples in the nMDS). These observations may be related to the origin of the index which, contrary to the Index of Trophic Diversity, was developed for soil and freshwater nematodes (Bongers and Bongers, 1998) and lately extended to assessing the condition of marine and brackish sediments, being less frequently applied to marine nematodes (Bongers et al., 1991), partly due to a lack of empirical support for the classification of some marine genera and the absence or rarity of extreme colonizers and persisters in most marine habitats (Schratzberger et al., 2006). According to Moreno et al. (2011), the analysis of the percentage composition of the different c-p classes in each site allowed a better classification of the studied sites than the application of the MI.

This study emphasized the need for the development of a nematode-based multimetric index (Patrício et al., 2012), taking in consideration density, composition, and genera sensitivity/tolerance to stress, as proposed by Moreno et al. (2011). Moreover, this multimetric index should include information with parameters more accurately based on marine/estuarine nematodes including maturity and trophic values specifically calculated for the genera. There is also the need for re-evaluation of the boundaries of the indices used, as an index can provide a good characterization of the system but may be limited to a specific spatial area. The correct application of nematode information and its integration into a multimetric index, with a suitable combination of several indicators, would provide clearer information regarding ecosystem status, since it would overcome the limitations of individual analyses. It is also important to bear in mind that the evaluation of reference conditions in order to provide comparisons with disturbed environments is usually required. Since meiobenthic studies are quite recent in Portuguese estuaries, it may be interesting to determine if the analysis of meiobenthic communities in an estuary where human perturbations are almost absent (Mira estuary – Alves et al., 2009; Adão et al., 2009) may be used in the establishment of reference conditions.

Chapter 3

Taxonomic resolution and Biological Traits Analysis (BTA) approaches in estuarine free-living nematodes

Taxonomic resolution and Biological Traits Analysis (BTA) approaches in estuarine free-living nematodes

ABSTRACT

The taxonomic and functional structure of the subtidal nematode assemblages from a temperate estuary (Mondego estuary, Portugal) was studied, focusing on different taxonomic levels (genus, family and order), on single functional groups and on multiple biological traits. Based on taxonomic levels and on four biological traits (feeding type, life strategy, tail and body shape), the analysis of the nematode assemblage distribution patterns revealed spatial differences but no clear temporal pattern. At the family and genus level, a separation of the upstream sections was observed, while a distinction of polyhaline and euhaline areas was less evident. The use of biological traits added new information regarding the relationships between diversity patterns and the environmental variables. Most nematodes encountered along the estuary were non-selective deposit feeders (1B) and omnivores/predators (2B), colonizer-persisters (score of 2 or 3), with clavate-conicocylindrical tails and slender bodies and with a distribution related essentially to salinity, oxygen and chlorophyll a. Applying a Biological Traits Analysis (BTA) showed the role of oxygen concentration in the distribution of the nematode communities. Although the BTA was no more powerful than the traditional taxonomic approach in detecting spatial differences along the Mondego estuary, it has increased our knowledge of the functional structure and characterization of nematode communities in the estuary.

Keywords: Free-living nematodes, taxonomic resolution, functional groups, Biological Traits Analysis (BTA), estuaries.

INTRODUCTION

Increasing pressures on marine ecosystems have been observed worldwide as a result of multiple natural and/or anthropogenic stressors (Dauvin, 2007). The need for scientific advice and legislation on ecosystem-based approaches to protect, conserve and manage the marine environment has never been greater (Schratzberger, 2012). It is essential that policy and decision-makers can effectively interpret the results of applied research, meeting the requirements of society for more comprehensive information regarding environmental issues (Lubchenco, 1998).

Among the biological components, meiobenthic communities can be a valuable tool to analyse the response to natural and disturbance gradients (Schratzberger, 2012). Free-living nematodes present several advantages for their use as monitoring organisms (Kennedy and Jacoby, 1999; Schratzberger et al., 2000; Alves et al., 2013). Besides being highly abundant, they play an important role as intermediaries between the microbial/detrital compartment and larger organisms (Danovaro et al., 2007) and their infaunal life style has a strong influence on the diversity and composition of the assemblage since they are intimately linked with the biogeochemical properties of the sediment (Heip et al., 1985; Steyaert et al., 1999). They could be considered the ideal model organism for exploring the relationship between biodiversity and ecosystem function (Danovaro et al., 2008), allowing to address key ecological issues, whether by using a taxonomic approach or by the analysis of biological traits.

The classical methods of nematode community analyses by the aggregation of species data into higher taxonomic groups appeared to reveal, according to Warwick (1988b), similar findings to those obtained by the analysis at the species level. Accordingly, Somerfield and Clarke (1995) examined the utility of estuarine nematodes in detecting impacts at higher taxonomic levels, concluding that aggregation to the level of genus produced robust interpretations, but not at higher levels. Similarly, for macrobenthic communities analyses at higher levels might more clearly reflect gradients being less affected by natural nuisance variables than species levels analyses. Although taxonomic sufficiency (the identification of

taxa to a level sufficient to permit the detection of changes in stressed assemblages; Ellis, 1985) still has criticism among the scientific community, particularly with respect to the potential losses of useful ecological information (Maurer, 2000), it allows the use of surrogate of species, such as higher taxonomic categories.

However, traditional taxonomic-based methods of nematode community analyses may not fully account for their diverse roles in ecosystem function (Schratzberger et al., 2007). It is recognized that changes in biodiversity may modify ecosystem function (Hooper et al., 2005) and taxonomic analyses alone may omit key functional aspects (Frid et al., 2000; Bremner et al., 2003). When attempting to evaluate the effects of environmental change, the inclusion of functional properties has been recommended (de Jonge et al., 2006).

According to Chalcraft and Resetarits (2003), species in functional groups share morphological traits that may represent an important ecological function. Free-living nematodes present several morphological characteristics thought to be related to important ecological functions: mouth structures (used as a proxy for feeding guilds, Wieser, 1953); tail shape (important in locomotion and reproduction, Thistle and Sherman, 1985; Thistle et al., 1995) and length-width ratio (adaptations to sedimentary environment; Jensen, 1987; Vanaverbeke et al., 2003; 2004). Furthermore, ecological characteristics such as life history strategy of nematodes (Bongers, 1990) can be informative of the condition of the habitats.

Biological Traits Analysis (BTA) takes the concept of functional groups further, aiming to describe function based on multiple traits (Bremner et al., 2003). BTA was recently applied to nematode communities of the southwestern North Sea area by Schratzberger et al. (2007). These authors used a set of five biological traits to investigate community function related to environmental variables.

Nematode assemblages have recently been studied along estuarine gradients in Portugal (Adão et al., 2009; Alves et al., 2013). In a previous study by Alves et al. (2013), the spatial and temporal biodiversity patterns of free-living nematodes in the Mondego estuary (NE Atlantic coast) were explored. Salinity and grain size composition proved to be important abiotic factors controlling the distribution of these assemblages. The present study builds on this study and

analyses both taxonomic and trait information of the subtidal free-living nematode communities in the Mondego estuary, to answer three questions: (*i*) How valuable are different taxonomic levels in detecting spatial and temporal distribution patterns? *(ii*) How valuable are single and multi-trait functional analyses in detecting these patterns? *(iii*) Is there added benefit in combining functional and taxonomic approaches?

MATERIALS AND METHODS

Study area

The Mondego estuary (Fig. 1), located on the western coast of Portugal (40° 08'N, 8°50'W), is a mesotidal system influenced by a warm-temperate climate. The estuary is a well-mixed system, some 21 km long with an area of approx. 8.6 km^2. In its terminal part (at a distance of 7 km from the sea) it divides into two arms, North and South, separated by an alluvial island (Murraceira island). The two arms have different characteristics (Marques et al., 1993). The North is deeper (5 - 10 m during high tide), receives most of the system's freshwater input and constitutes the main navigation channel supporting the Figueira da Foz harbour. The South is shallower (2 - 4 m during high tide), covered by large areas of intertidal mudflats (75% of the area). The estuary supports several industries, salt-works, agricultural areas, mercantile and fishing harbours, having various anthropogenic pressures (Marques et al., 1993; Flindt et al., 1997).

Sampling strategy, laboratory procedures and data sets

Nematode communities were sampled on six occasions: August 2006 (Au06), November 2006 (Nv06), March 2007 (Mr07), June 2007 (Ju07), September 2009 (Sp09) and December 2009 (Dc09); at eleven stations along the estuary (Fig. 1). Stations were selected following the estuarine division proposed by Teixeira et al. (2008) based on the main water and sediment variables (salinity, sediment grain size composition and organic matter content) structuring benthic communities within the estuary. Five different areas covering this natural variability were sampled: Euhaline (station 4), Polyhaline South arm (stations 6, 7

and 9), Polyhaline North arm (stations 12 and 13), Mesohaline (stations 18 and 19) and Oligohaline (stations 21, 23 and 25) (Fig. 1).

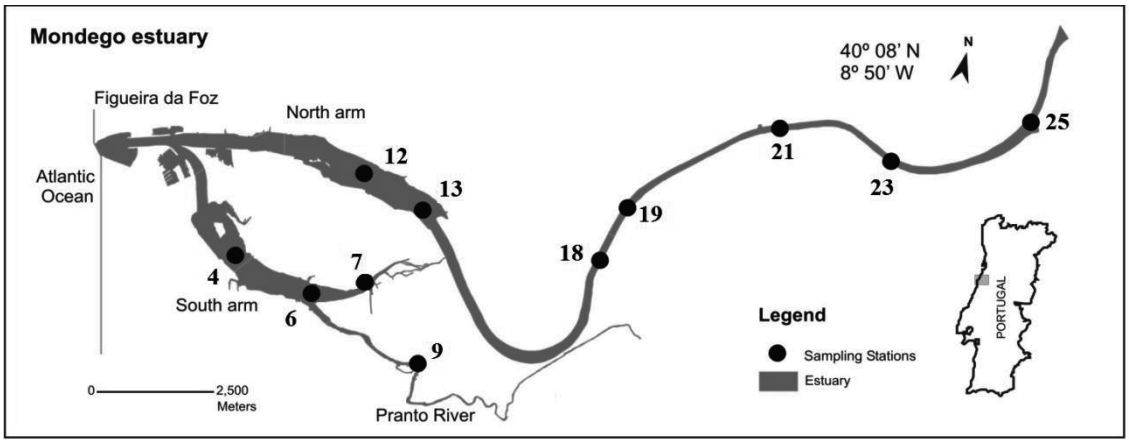


Figure 1. Mondego estuary (Portugal). Station locations represented by the black circles. Estuarine areas: Euhaline (station 4), Polyhaline South arm (stations 6, 7 and 9), Polyhaline North arm (stations 12 and 13), Mesohaline (stations 18 and 19) and Oligohaline (stations 21, 23 and 25).

Environmental data

Bottom water variables were measured *in situ* at each station, using an YSI Data Sonde Survey 4: salinity (except for December 2009), and dissolved oxygen (mg L^{-1}). Additionally, water samples were collected for laboratory determination of dissolved nutrients concentration and chlorophyll *a* (mg m^{-3}). Nitrates (NO_3^--N), nitrites (NO_2^--N), ammonia (NH_4^+-N) and phosphates (PO_4^{3-}-P) concentration (µmol L^{-1}) were analysed as described in Strickland and Parsons (1972) and in Limnologisk Metodik (1992). Chlorophyll *a* determinations were performed according to Parsons et al. (1985).

Sediment samples were also taken at each station to determine organic matter content and grain size distribution. Organic matter content was estimated as the difference between the dry sediment (at 60ºC for 72 h) and the sediment weight after combustion (450ºC for 8 h), and expressed as a percentage of total sample weight. Grain size analysis was performed by dry sieving through a column of sieves with different mesh sizes and the classification system of Brown and McLachlan (1990) was followed (gravel: >2 mm; coarse sand: 0.500–2.000 mm; medium sand: 0.250–0.500 mm; fine sand: 0.063–0.250 mm; and silt and clay:

<0.063 mm). The relative amount of the different grain-size fractions was expressed as a percentage of total sample weight (Annex 2).

Nematode data

At each station, three replicates of subtidal sediment were collected, by inserting a Kajak corer (inner diameter: 4.6 cm) 3 cm into the sediment. To extract the meiofauna, the sediment cores were then sieved through 1 mm and 38 µm mesh size sieves and the fraction retained in the 38 µm sieve centrifuged in Ludox HS-40 colloidal silica at a specific gravity of 1.18 g cm^{-3} (Vincx, 1996). The supernatant was rinsed with water and stored in a 4% buffered formalin solution. Nematodes were counted under a stereomicroscope and, from each replicate, 120 nematodes (if present) were picked out randomly and mounted on glycerin slides (Vincx, 1996). Specimens were identified to genus level using a microscope (maximum magnification 1000x) and the keys of Platt and Warwick (1983; 1988), Warwick et al. (1998), Abebe et al. (2006) plus the online information system 'NeMys' (Steyaert et al., 2005). Family and order classification followed the classification of Lorenzen (1981) including modifications proposed by Platt and Warwick (1983; 1988). Freshwater nematodes followed the classification proposed by Abebe et al. (2006) based on De Ley and Blaxter (2004).

Biological Traits Analysis (BTA)

Information for assigning each taxon to a functional group was obtained from various published sources (Platt and Warwick, 1983, 1988; Warwick et al., 1998; Steyaert et al., 2005; Abebe et al., 2006). The traits selected were:

(a) **Feeding type:** following Wieser (1953), and based on the buccal cavity morphology, nematodes were classified as: selective deposit feeder (1A), non-selective deposit feeder (1B), epigrowth feeder (2A) and omnivore/predator (2B).
(b) **Life strategy:** following Bongers (1990) and Bongers et al. (1991), taxa were classified on the c-p scale, ranging from 1 (extreme colonizers: short life cycle, high reproduction rates, tolerant to various types of disturbance) to 5 (extreme persisters: long life-cycles, few offspring, sensitive to disturbance).

(c) **Tail shape:** following Thistle et al. (1995), tail shape was classified as rounded (with a blunt end), clavate-conicocylindrical (initially conical with an extension to the tip), conical (with a pointed tip) and long (a tail longer than five body widths).
(d) **Body shape**: following Soetaert et al. (2002), nematode morphology was classified as: stout, slender and long/thin.

After the traits selection, BTA computation followed the procedures described by Bremner et al. (2003; 2006a). In essence, three different numerical matrices are required: (1) "taxa by station" (taxa density in each station); (2) "taxa by traits" (biological traits for each taxon); and (3) "traits by station" (biological traits in each sampling station; the cross-product of the previous two matrices). The final "traits by station" data matrix was achieved by multiplying trait categories for each taxon present at a station by its density at that station, and then summing over all taxa present at each station to obtain a single value for each trait category in each sample (Bremner at al., 2006b). To perform the analysis, R environment was used (R Development Core Team, 2009) and the resulting 'traits by station' data matrix was subjected to multivariate analysis.

Data analysis

Multivariate analyses of biological and environmental data were performed using PRIMER v6 software package (Clarke and Gorley, 2006) with the PERMANOVA add-on (Anderson et al., 2008).

Environmental data

A Principal Components Analysis (PCA) of the environmental variables was performed. The redundant variables were removed from the analysis so that the first two axis account for the maximum variability in the dataset. The variables retained in the model act as proxy for the ones that were eliminated. Prior to the calculation of the resemblance matrix using the Euclidean distance coefficient, variables were square root transformed (salinity, ammonia, chlorophyll *a*, silicates, organic matter, mean sand and gravel), to reduce the right asymmetry of data distribution (with the exception of dissolved oxygen) and then normalized. The relationships between environmental variables and the taxonomic (genus, family

and order) and functional structure (single functional groups and combined biological traits matrix resulting from BTA) of nematode communities, were explored by carrying out BIOENV analyses (Clarke and Ainsworth, 1993). Spearman's rank correlations were used and a permutation test was applied to assess the significance of these relationships.

Nematode assemblages

Tests of spatial and temporal differences were carried out using two-way permutational multivariate analyses of variance (PERMANOVA). All PERMANOVA analyses were performed using a crossed factor experimental design: "area" and "sampling occasion" as fixed factors, with five (Euhaline, Polyhaline North arm, Polyhaline South arm, Mesohaline and Oligohaline) and six (August 2006, November 2006, March 2007, June 2007, September 2009 and December 2009) levels, respectively. The 'Permutation of residuals under a reduced model' option was selected and 9999 permutations carried out. When significant differences ($p<0.05$) were detected, these were further examined using *a posteriori* pair-wise comparisons.

To visually assess spatial and temporal patterns, non-metric Multidimensional Scaling (nMDS) ordinations were carried out. Data were first square root transformed and the Bray-Curtis coefficient was the similarity coefficient used. The Similarity Percentage Analysis (SIMPER) was used to determine which taxa contributed most to similarity within areas and to dissimilarity between them (cut-off 75%). Resemblance (correlation) matrices derived from each taxonomic level, single trait groups and multi-trait matrix were then used in a second-stage nMDS analysis to examine similarities among each of the first-stage MDS matrices (Somerfield and Clarke, 1995), by means of Spearman's rank correlations.

RESULTS

Environmental variables

The first two PCA axes accounted for 60.8% of the total variation (Fig. 2). A clear separation of sampling areas was shown (Fig. 2A): the euhaline and polyhaline NA areas presented higher salinity and medium size particles diameter; the polyhaline SA was characterized by higher organic matter content and fine sediments whilst both mesohaline and oligohaline upstream areas were distinguished by higher nutrient concentration and chlorophyll *a* content. In turn, temporal distinction was not evident (Fig. 2B) although samples from Sp09 and Dc09 presented mainly fine sediments, high organic matter content and nutrients concentrations. In summary, the spatial gradient appeared clearer than the temporal one.

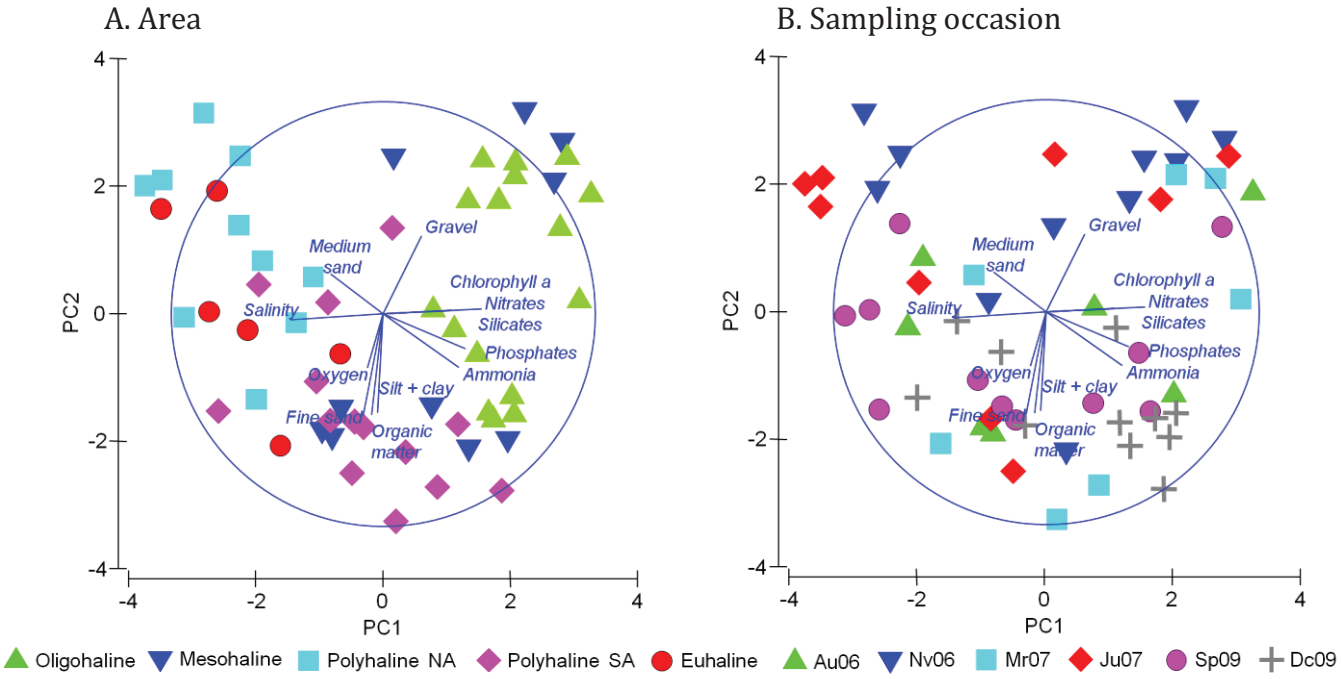


Figure 2. Principal Components Analysis (PCA) plot based on the environmental variables in each A) "area" (Euhaline, Polyhaline North arm, Polyhaline South arm, Mesohaline and Oligohaline) and B) "sampling occasion" [August 2006 (Au06), November 2006 (Nv06), March 2007 (Mr07), June 2007 (Ju07), September 2009 (Sp09) and December 2009 (Dc09)]. PC1=32.8%, PC2=28.0%.

Taxonomic classification

When considering taxonomic classification, significant spatial and temporal differences at each level (genus, family and order) were detected by the two-way PERMANOVA analyses (all $p<0.05$; Annex 3 and 4). A clear spatial segregation of the oligohaline and mesohaline areas from the remaining was observed in nMDS ordination plots regardless of the taxonomic level analysed (Fig. 3 A-C), highlighting the particular species composition of the nematode assemblages inhabiting these areas. The SIMPER analysis (Annex 5) showed that these areas were mainly characterized by the genera *Daptonema*, *Mesodorylaimus*, *Ptycholaimellus*, *Anoplostoma*, *Sabatieria*, *Dichromadora*, *Paracyatholaimus*, *Viscosia*, *Neotobrilus*, *Mononchus*, *Terschellingia*, *Plectus*, *Axonolaimus*, *Theristus* and *Eudorylaimus* (oligohaline area), and *Daptonema*, *Anoplostoma*, *Dichromadora*, *Terschellingia*, *Viscosia*, *Paracyatholaimus*, *Sabatieria*, *Ptycholaimellus*, *Sphaerolaimus* and *Leptolaimus* (mesohaline area).

In turn, the Euhaline area presented no significant differences in species composition over time for the various taxonomic levels. This section was mainly characterized by the genera *Daptonema*, *Sabatieria*, *Viscosia*, *Sphaerolaimus*, *Linhomoeus*, *Oncholaimellus*, *Dichromadora*, *Anoplostoma*, *Terschellingia*, *Molgolaimus*, *Paracyatholaimus*, *Odontophora*, *Ptycholaimellus*, *Metachromadora*, *Halalaimus*, *Chromadorita* and *Microlaimus*, belonging to the families Xyalidae, Comesomatidae, Oncholaimidae, Spaherolaimidae, Linhomoeidae, Chromadoridae, Desmodoridae, Axonolaimidae, Anoplostomatidae and Cyatholaimidae. There was no obvious temporal pattern for each taxonomic level considered in assemblage composition (Fig. 3 D-F).

Biological traits: spatial and temporal patterns

With regard to the biological traits characterizing each estuarine zone during the study period, the different traits varied in their spatial and temporal distribution (Fig. 4 A-D). Overall, assemblages were dominated by non-selective deposit feeders (1B, 50.5%) and omnivores/predators (2B, 20.9%) (Fig. 4A). Most nematodes attained a colonizer-persister score of 2 or 3 (cp=2: 68.1%, cp=3: 27.8%), while scores of 1 or 5 were rare (Fig. 4B). Clavate-conicocylindrical and

conical tails were the prevalent tail shapes (55.8% and 23.2%, respectively; Fig. 4C) and, from the three body shapes analysed, a predominance of slender bodies (96.7%) was observed (only 3.2% of nematodes presenting long/thin bodies) (Fig. 4D). When considering the biological traits composition data, significant spatial and temporal differences for single traits and for the multi-trait approach were detected by the two-way PERMANOVA analyses (Annex 3 and 4). It is of note that there were no temporal differences in the polyhaline NA area. These patterns can be observed in the nMDS plots, where the spatial segregation of the oligohaline area is visible (Fig. 5 A-E) but with no obvious temporal patterns (Fig. 5 F-J).

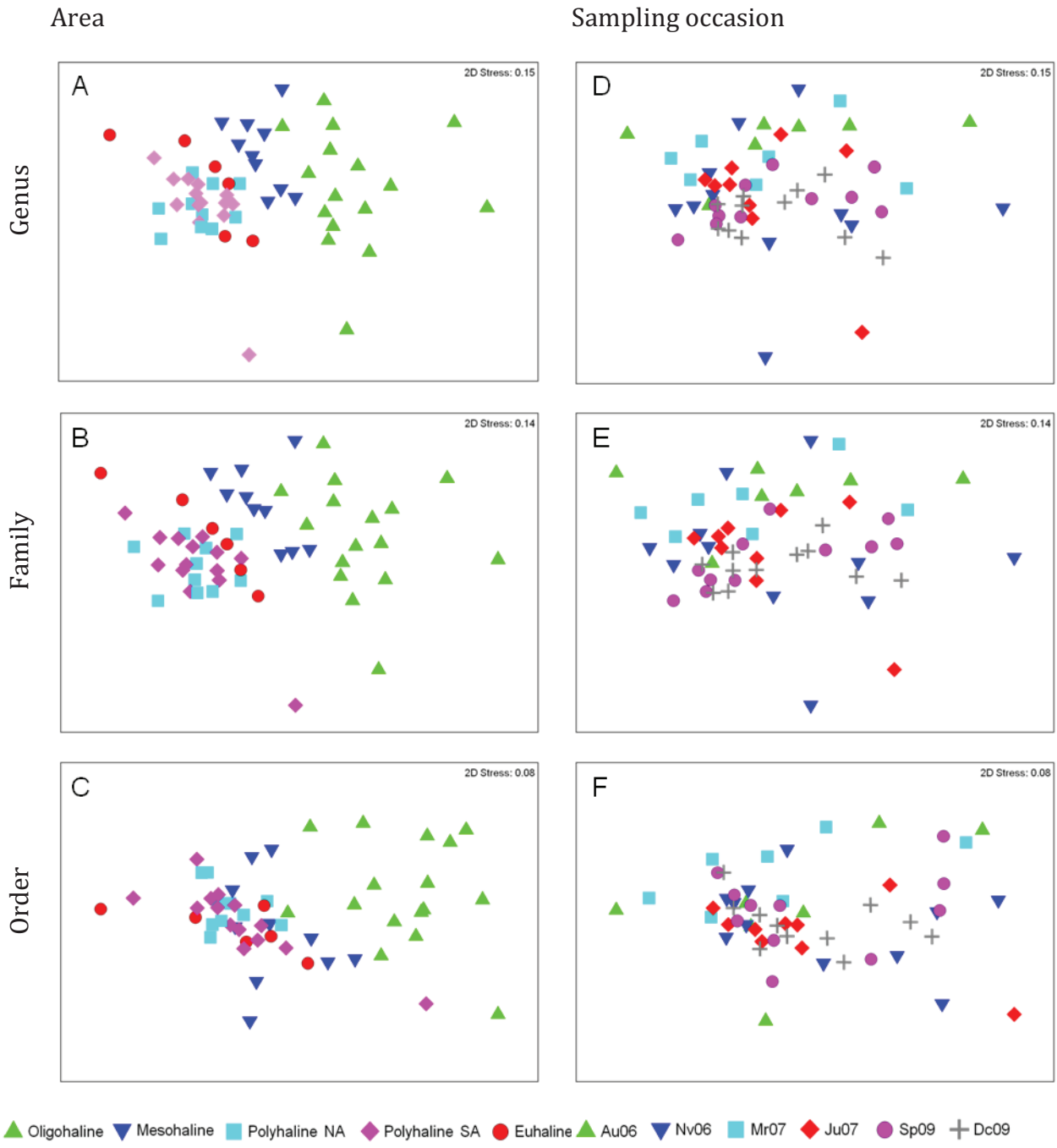


Figure 3. nMDS ordination plots of nematode abundance at each taxonomic level (genus, family and order), coded for the spatial factor "area" (Euhaline, Polyhaline South Arm, Polyhaline North Arm, Mesohaline and Oligohaline) (A, B, C) and for the temporal factor "sampling occasion" [August 2006 (Au06), November 2006 (Nv06), March 2007 (Mr07), June 2007 (Ju07), September 2009 (Sp09) and December 2009 (Dc09)] (D, E, F).

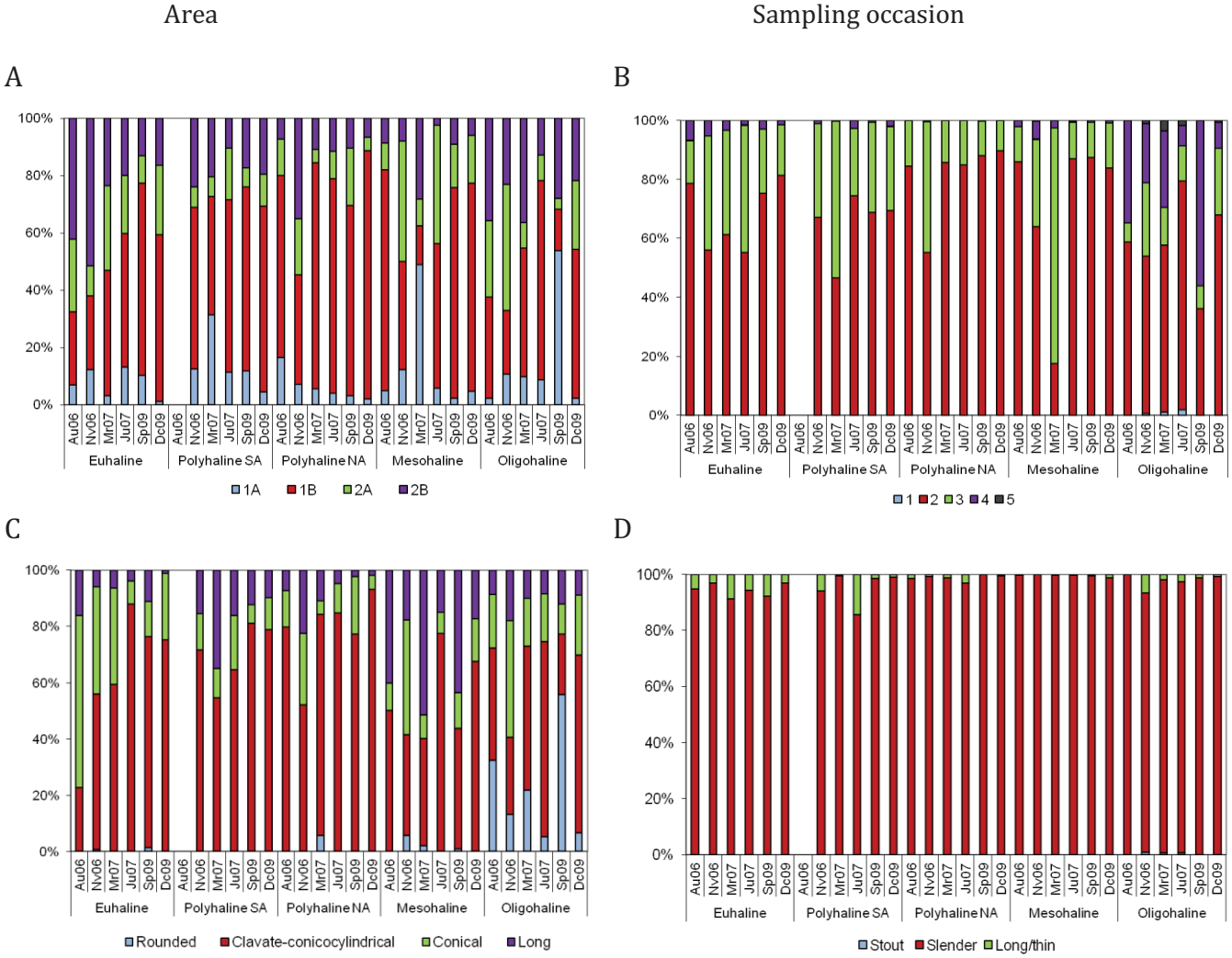
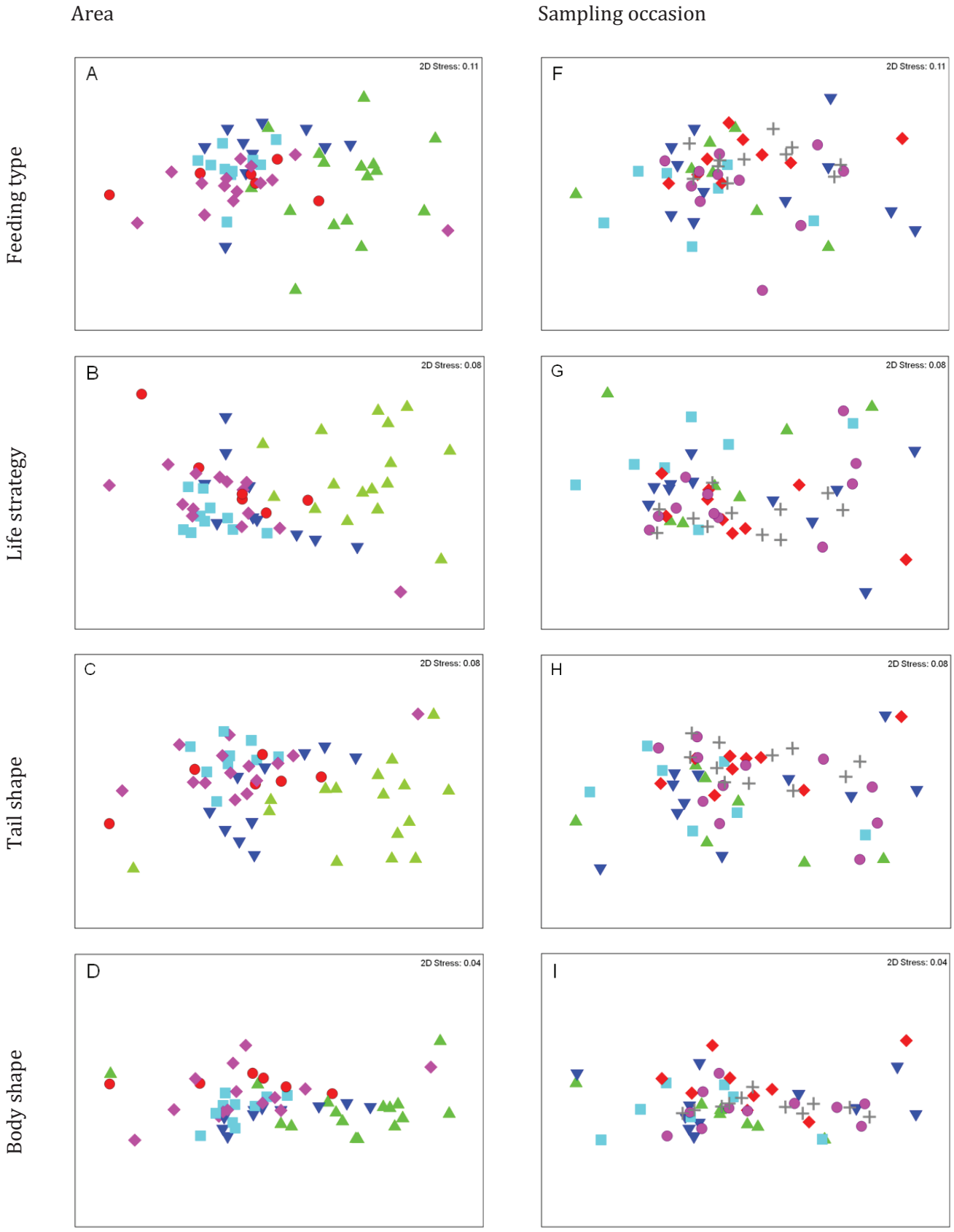


Figure 4. Biological traits patterns along the estuarine gradient and over time. Areas: Euhaline, Polyhaline South Arm, Polyhaline North Arm, Mesohaline and Oligohaline); Sampling occasions: August 2006 (Au06), November 2006 (Nv06), March 2007 (Mr07), June 2007 (Ju07), September 2009 (Sp09) and December 2009 (Dc09). Biological traits: (A) Feeding type, (B) Life strategy, (C) Tail shape and (D) Body shape.



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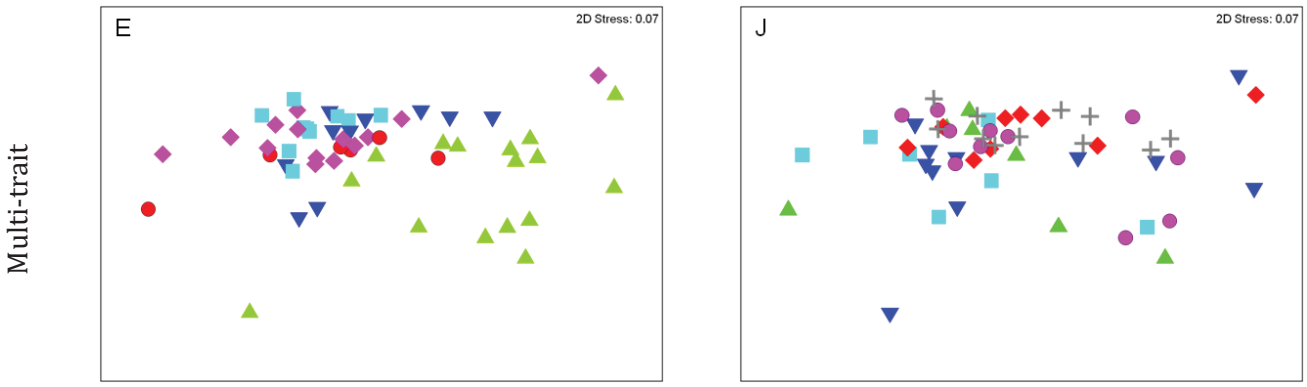

Figure 5. nMDS ordination based on biological traits information (single functional groups and multi-trait) at each "area" (Euhaline, Polyhaline South Arm, Polyhaline North Arm, Mesohaline and Oligohaline) (A to E) and "sampling occasion" [August 2006 (Au06), November 2006 (Nv06), March 2007 (Mr07), June 2007 (Ju07), September 2009 (Sp09) and December 2009 (Dc09)] (F to J).

Taxonomic and functional composition

Combining the information from both taxonomic and functional approaches, the 2nd stage nMDS plot (Fig. 6) revealed that biological traits information differed from the taxonomic information, since biological traits clustered together, clearly separated from taxonomic levels. Multi-trait data clustered closest to single traits than to taxonomic levels data. Results from the BIOENV analyses showed that, although low correlation values were obtained, the distribution of nematodes at the different taxonomic levels was mainly related to salinity, nutrients and chlorophyll *a*. The main structuring factors of the trait distribution were salinity, oxygen, nitrates, grain size (fine sand and gravel) and chlorophyll *a* (Table 1).

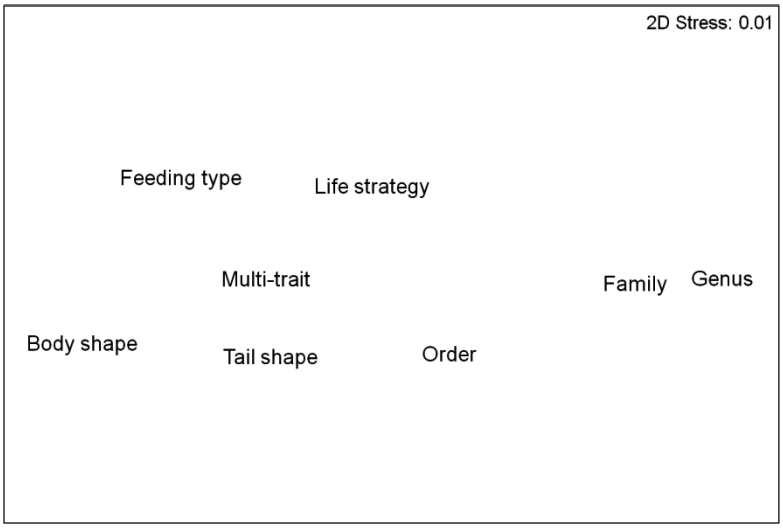

Figure 6. Second stage non-metric MDS plot of inter-matrix Spearman correlations among matrices of taxonomic levels (genus, family and order), single traits composition (feeding type, life strategy, tail shape and body shape) and multi-trait data.

Table 1. Results from BIOENV analyses: Spearman rank correlation (*rho*) and significance level (*p*) between nematode data (taxonomic levels and biological traits) and environmental variables. Values in bold were significant at $p < 0.05$.

	rho	*p*	Environmental variables
Genus	0.419	**0.01**	Nitrates, silicates, gravel, chlorophyll *a*
Family	0.402	**0.01**	Oxygen, nitrates, silicates, gravel, chlorophyll *a*
Order	0.352	**0.01**	Salinity, nitrates, silicates, fine sand, chlorophyll *a*
Feeding type	0.228	**0.02**	Salinity, silt+clay, fine sand, gravel, chlorophyll *a*
Life strategy	0.318	**0.01**	Salinity, nitrates, silt+clay, fine sand, chlorophyll *a*
Tail shape	0.287	**0.01**	Salinity, oxygen, nitrates, fine sand, chlorophyll *a*
Body shape	0.201	0.9	Oxygen, nitrates
Multi-trait	0.282	**0.01**	Salinity, oxygen, nitrates, fine sand, chlorophyll *a*

DISCUSSION

By describing the taxonomic and functional structure of nematode assemblages in the Mondego estuary and by contrasting the information provided when using different approaches, the present study highlighted the importance of the estuarine spatial gradient in driving the distribution of the taxonomic and functional groups. To address the most relevant findings from the analysis of the

subtidal nematode communities, this Discussion is divided according to the three main research questions initially posed.

Taxonomic classification

Taxonomic sufficiency has received much attention in assessment studies, especially in freshwater systems, mainly due to logistical difficulties, cost and time involved in species-level identification (Trigal-Domínguez et al., 2010). However, despite the advantages of a coarser resolution, in impact assessment studies and perturbation gradients a finer resolution can be desirable to reveal differences in the community structure (Trigal-Domínguez et al., 2010). The spatial and temporal analysis of the nematode assemblage data at different taxonomic levels in the Mondego estuary revealed a clear spatial segregation of the communities. Less obvious was the temporal effect on the distribution pattern of the communities. These findings agree with Alves et al. (2013) who gave a detailed account of the genus distribution patterns, diversity and community structure of the nematode communities in the Mondego estuary. A predominance of the spatial effect over the temporal one on the distribution patterns of assemblages was also observed. At both genus and family level, a clear separation of the upstream areas (mesohaline and oligohaline) was observed, due to dominance of typical freshwater communities in these areas. On the other hand, at the order level, spatial differences were not clear.

Salinity is an important environmental factor influencing nematode distribution within the estuaries (Heip et al., 1985; Austen and Warwick, 1989; Soetaert et al., 1995). In this study, salinity together with sediment composition, were the most important abiotic factors distinguishing nematode genera and family patterns within the estuary. Fewer factors were important for describing order-level assemblage patterns.

Somerfield and Clarke (1995) have highlighted that analyses of sublittoral and intertidal nematode communities are robust to aggregation to the level of genus, but further aggregations start to alter the perceived patterns of impact. Although no direct anthropogenic impact was analysed in the present study, the nematode distribution patterns along the estuarine natural gradient also revealed

clear at lower taxonomic levels than order-level. Therefore, for this particular system, analyses using taxonomic resolutions at genus or family level seem advantageous to highlight community distribution patterns, which is important when implementing future management actions.

Biological traits

Single traits

The feeding characterization of nematodes confirmed, at the spatial level, the separation of the oligohaline area from the remaining estuarine areas, mainly due to the high percentage of predators. With the exception of the euhaline area, where both non-selective deposit feeders (1B) and omnivores/predators (2B) were present at similar densities, non-selective deposit feeders dominated in each area and on various sampling occasions. Similar dominance patterns of non-selective deposit-feeders nematodes were observed by Schratzberger et al. (2007; 2008) in the North Sea. However, this dominance can be questionable since, according to several authors that have revised and modified Wieser's classification (Romeyn and Bouwman, 1983; Jensen, 1987; Moens and Vincx, 1997; Moens et al., 2004), confining species to a single trophic role may not represent the real plasticity in changing feeding strategies observed in several nematodes (Moens et al., 2005; Schratzberger et al., 2008) as a response to the complexity of the available feeding habitats (Moens and Vincx, 1997). Furthermore, the trophic plasticity has also been suggested as responsible for the absence of temporal relations between the trophic nematodes composition and food availability (chlorophyll *a* or carbon sedimentation) (Schratzberger et al., 2008).

According to Bongers at al. (1991), the life strategy characterization provides important additional information to that given by the feeding types regarding disturbance. A different composition was observed in both euhaline and polyhaline SA areas, where a dominance of colonisers and intermediate (c-p 2 and 3) taxa was registered, suggesting a high stress level with an increase of opportunistic genera. Higher abundance of coloniser nematodes was even more obvious at the polyhaline NA area, pointing to a disturbed condition. However,

whether this high abundance of colonisers is caused by disturbance, increases in decomposition or in quantity of food (favouring fast-reproducing species) (Bongers et al., 1991) is not easily determined. Despite this, Moreno et al. (2011) suggested including c-p class percentage as an ecological quality indicator, since reliable results regarding environmental conditions (previously defined in sediments of the Mediterranean sea) were obtained considering the different percentage composition of c-p classes.

Assuming that similar shapes correspond, to a certain extent, to similar fitness constraints, morphometric characterization becomes a useful descriptor of ecosystems (Schwinghamer, 1983). Nematode tails play an important role in the locomotion, feeding and reproduction processes and morphological adaptations are characteristic of specific environments (Thistle and Sherman, 1985). The four types analysed showed a dominance of clavate-conicocylindrical tails along the estuary, especially in the polyhaline areas, while long tails were abundant on the mesohaline area. Long tails were reported by Riemann (1974) for individuals that have a partly sessile existence in which tail morphology plays a crucial role, especially in sand (Ax, 1963) and muddy sediments (Riemann, 1974), enabling animals to retract from blocked interstitial passageways and forage for food. In agreement, this estuarine area was characterized by relatively small particle diameter (medium sand). The abundance of conical tails in the euhaline area points towards a different structure of the community. According to Thistle et al. (1995), insights based on tail shape give additional information to that incorporated by the buccal-morphology groups, making them potentially useful as ecological indicators.

Losi et al. (2013) found nematode body shape to be an informative parameter which was suggested to be related with the available food and biogeochemical conditions of the sediment (Tita et al., 1999; Soetaert et al., 2002; Vanaverbeke et al., 2004). This trait was the least informative regarding the separation of areas since slender bodies dominated in all areas and sampling occasions, not presenting any clear relation with the environmental factors analysed. However, stout nematodes appeared mainly in the oligohaline area, which can be related to the lower values of oxygen in this section. According to

Soetaert et al. (2002), depth in the sediment influences the length and width of nematodes, being consistent with an adaptation to changing oxygen concentration, with nematode body width decreasing simultaneously, resulting in higher oxygen absorption efficiency. On the other hand, long/thin nematodes were found in the downstream areas (euhaline and polyhaline areas), which could be hypothesized to be related with a more unstable environment, since this body shape is thought to be advantageous for "hanging" in high-energy or coarse-sediment habitats (Gerlach, 1953; Wieser, 1959; Warwick, 1971; Tietjen, 1976; Thistle and Sherman, 1985).

Multi-trait

Assigning the functional traits to each nematode genus may lead to a reduction of a generally high diversity into a small number of single functional groups (suggesting limited functional diversity), resulting in the underestimation of the true functional complexity of nematode communities (Thistle et al., 1995; Schratzberger et al., 2007). In turn, combining multiple biological traits expressed by the organisms has been considered a more reliable approach in assessing functional structure of nematode communities (Schratzberger et al., 2007).

The distribution pattern of the communities based on the BTA approach was similar to that observed with the single traits, although it has proved not a simple reflection of the information contained in the latter. Similar findings were also reported by Schratzberger et al. (2007) for nematode communities in the southwestern North Sea.

The merger of the functional features represents a more realistic approach, since different aspects of the functioning of the system are gathered. For instance, nematodes within the same trophic group present a wide range of life strategy categories and Postma-Blaauw et al. (2005) showed that differences in life history strategies between nematode species of the same trophic group is of importance for their communal effect on soil ecosystem processes.

Along the Mondego estuary, in addition to the main environmental variables that are known to influence nematodes distribution in the sediments (salinity and grain size), dissolved oxygen appeared an important factor related to community

distribution. This variable is mostly referred as structuring the vertical profile of nematodes in the sediment, since the vertical distribution of diversity and density of nematodes is related with the penetration of dissolved oxygen (Coull, 1999; Soetaert et al., 1994). The recognition of dissolved oxygen as a structuring factor of nematode assemblage distribution in the Mondego estuary became most apparent when applying BTA. Since the most abundant genera found (*Terschellingia*, *Sabatieria* and *Daptonema)* are known to be typical of poorly oxygenated and organically enriched bottoms (Soetaert et al., 1994; Schratzberger et al., 2006; Steyaert et al., 2007), this suggests some degree of system disturbance.

The information on biological traits is still scarce for free-living nematodes and the affinity of each genus to each trait category is not easily assigned, as for macrobenthic communities. For the latter communities a wide range of information is available and the extent a species expresses each category (there might be variability with respect to traits that vary over species' life cycles or between populations – Bremner, 2008) can be defined, using procedures such as 'fuzzy coding' (Chevenet et al., 1994). Due to lack of information on nematodes, equal weighting to all traits had to be considered in this study. As pointed out by Schratzberger et al. (2007), there is still a need for greater knowledge regarding functional roles of nematodes, which will help interrogate the sensitivity and interpretation of biological traits analyses.

Taxonomic vs. functional approaches

Despite the fact that different communities characterize different areas of the estuary and variation in the categories of each trait along the estuarine gradient have been observed, the dominance of some traits was consistent along the system, suggesting functional maintenance. According to Walker et al. (1999) and Warwick and Clarke (2001) changes in phylogenetic diversity of species assemblages are not explicitly linked to changes in functional diversity and so their ecological significance can be difficult to assess.

The biological traits approach, while of value, was no more powerful than the traditional taxonomic approach in detecting spatial differences along the Mondego estuary. Similar outcomes were observed by Schratzberger et al. (2007)

for nematode assemblages in the North Sea and Armenteros et al. (2009) in the Caribbean Sea, where the inclusion of trait-based analyses provided additional information of community distribution patterns regarding different environmental factors. In the present study although the information obtained by the taxonomic approach was not superimposed on that obtained with the functional ones, the distribution patterns of the communities were related to similar sets of environmental parameters. Nevertheless, trait-based approaches contributed to increase knowledge on the functional structure and characterization of nematode communities in the estuary.

The use of biological traits has been strongly encouraged in studies aiming at analysing diversity patterns (Armenteros et al., 2009) and assessing ecosystem functioning (Bremner et al., 2003). In this context, since trait-based approaches are known for their high robustness with decreased taxonomic resolution (Menezes et al., 2010), problems associated with misidentification can be less critical since nematode species with high morphological similarity will most probably share the same trait category.

CONCLUSIONS

A characterization of the traits structure was performed, for the first time, for the nematode communities of the Mondego estuary. No clear temporal pattern was observed in traits distribution and considering different taxonomic levels, while spatial differences were evident using both taxonomic and functional approaches. Genus and family identification level allowed similar outcomes regarding spatial differentiation of estuarine areas with a clear separation of the upstream oligohaline and mesohaline areas due to their particular species composition. The single-trait approach also highlighted the peculiarity of the upstream areas and the multi-trait approach emphasised the importance of specific environmental factors (oxygen and nutrients) on the distribution patterns of the nematode communities along the estuary. This shows the value of the application of traits-based methods, providing complementary types of information to that obtained by the classical taxonomic methods.

Chapter 4

Estuarine intertidal meiofauna and nematode communities as indicator of ecosystem's recovery following mitigation measures

Estuarine intertidal meiofauna and nematode communities as indicator of ecosystem's recovery following mitigation measures

ABSTRACT

The Mondego estuary (Portugal) has been under environmental pressure since the early 1990's due to different anthropogenic stresses. The system has been studied following benthic communities' features from an impacted situation until the recovery phase, focusing mostly on macrobenthos.

Following the application of mitigation measures in the estuary, this study analyzed the intertidal meiobenthic and nematode communities' distribution patterns at the temporal and spatial levels to assess their changes as a response to the restoration efforts. Results pointed towards a similarity between the areas (with variations being attributed to factors usually related with estuarine communities' distribution), suggesting that the system has recovered from the early situations.

To the best of our knowledge this is the first attempt to investigate the variability of intertidal meiobenthic and nematode communities in the scope of a system's recovery along an estuarine gradient of eutrophication, revealing the effectiveness of the mitigation measures applied.

Keywords: intertidal meiobenthos, free-living marine nematodes, ecological quality assessment, estuaries, ecosystem recovery.

INTRODUCTION

Estuaries are dynamic and productive systems (Kennish, 2002), being amongst the most valuable ecosystems in the world (Costanza et al., 1997). Besides supporting important ecological functions and services (e.g. biogeochemical cycling and movement of nutrients, water purification, flux regulation of water, particles and pollutants, shoreline protection) (Kennish, 2002; Meire et al., 2005; Paerl, 2006), resources provided by estuaries have been a target of human exploitation, compromising estuarine ecological integrity (Halpern et al., 2008; Borja et al., 2010). Furthermore, human induced impacts (including nutrient enrichment, chemical contamination, hydrological modification, habitat loss, among others; Kennish, 2002) and their negative effects on estuarine systems triggered the attention toward the need for monitoring, assessing and managing ecological integrity to promote the long-term sustainability of these systems (Borja et al., 2008).

Estuarine communities have to cope with the high variability in the physicochemical characteristics felt within these systems (Elliott and Quintino, 2007) and this natural variability may confer them an ability to withstand stress (positive effects on organisms able to tolerate adverse and variable conditions, capitalizing the lack of inter-specific competition), both natural and anthropogenic, increasing the difficulty in detecting a signal reflecting anthropogenic change in estuaries (Estuarine Quality Paradox) (Elliott and Quintino, 2007). Establishing relationships between species distribution and environmental characteristics is a major goal in the search for forces/causes driving species distribution (Peres-Neto et al., 2006) and the awareness of increasing pressures on aquatic systems enhanced the development and implementation of environmental policies worldwide, addressing the ecological quality or integrity within estuarine systems (Borja et al., 2008).

Regarding environmental assessments, good indicators are those that respond to natural gradients or disturbance at spatio-temporal scales appropriate to the study and faunal groups are deemed appropriate for this task (Schratzberger, 2012). Although macrobenthic invertebrates are favored as

indicators in aquatic assessments over meiofauna (mainly due to well documented sampling protocols and taxonomic keys for macrobenthos), meiofauna are useful indicators in a variety of studies (their close association with the substrate, high diversity and importance in ecosystem functioning makes meiofauna a valuable tool for environmental assessments) (Heip et al., 1985; Sandulli and de Nicola, 1991; Kennedy and Jacoby, 1999; Schratzberger et al., 2000; Moreno et al., 2008).

Community-based approaches in estuaries relate the horizontal distribution of meiobenthos and nematode communities at different scales (from small to global scale) with the complex interaction between biotic (food source distribution, competition among species) (Montagna et al., 1983; Galluci et al., 2008) and abiotic factors (variations in the physicochemical properties of the sediment matrix, salinity and tidal exposure) (Heip et al., 1985; Steyaert et al., 2001; Steyaert et al., 2003; Ferrero et al., 2008). Moreover, human disturbances affecting the physical structure of the sediment and food availability, as well as pollution impacts on nematode communities have been documented (Coull and Chandler, 1992; Schratzberger and Warwick, 1999b; Schratzberger et al., 2000; 2002), reinforcing nematode communities as highly informative and useful in efficiently evaluate the ecological status of aquatic bodies (Moreno et al., 2011).

The Mondego estuary (Portugal), a south-western European transitional system, underwent intense anthropogenic pressure over the last decades, promoting an overall decline in its environmental quality (*further description in Materials and Methods*). Following a management measure in the Spring of 2006 (Veríssimo et al., 2012a; 2012b), it was created the opportunity to assess and compare the system new ecological quality status with the previous eutrophication state, and studies relating these conditions were especially performed for macrobenthic communities (Veríssimo et al., 2012a; 2012b; Marques et al., 2013).

Regarding meiofauna and nematode communities, data previous to the intervention are not available. However, due to the extensive knowledge regarding the system evolution in the South arm of the Mondego estuary (spatial gradient of eutrophication – Marques et al., 1997; *see Materials and Methods*), the analysis of meiofauna communities' succession can give new insights about the system recovery. Following a gradient of *Zostera* coverage, this study has as main goals: ***i***)

the analysis of changes in intertidal meiofaunal communities, especially free-living nematodes, along an eutrophication/recovery gradient, ***ii***) the identification of relations between the obtained distribution patterns and the physicochemical environment, and ***iii***) the interpretation and integration of the results considering the evolution (recovery) of the system, in order to understand how nematode communities reflect the impacts. We hypothesized that ***i***) meiofauna and nematode communities will be different along the south arm of the Mondego estuary, with higher diversity and abundance in the area dominated by *Zostera noltii*, and that ***ii***) the differences between areas can be attributed to the different pressures suffered during time at the different areas.

MATERIALS AND METHODS

Study area

The Mondego estuary, located in the western coast of Portugal (40° 08'N, 8 ° 50'W) (Fig. 1), is a mesotidal system influenced by a warm-temperate climate. In its terminal part this 21 km long estuary consists of two arms – north and south - separated by an alluvial island, and join again in the estuary mouth. The two arms present different hydrological characteristics (Marques et al., 1993; Marques et al., 2003): the south arm is shallower (2-4 m during high tide), covered by large areas of intertidal mudflats (almost 75% of the area) exposed during low tide (Neto et al., 2008); the north arm is deeper (5-10 m during high tide), receives most of the system's freshwater input and constitutes the main navigation channel supporting the Figueira da Foz harbour. The estuary supports several industries, salt-works, agricultural areas, mercantile and fishing harbours, thus having various anthropogenic pressures (Marques et al., 1993; Flindt et al., 1997).

The estuary has suffered several physical modifications over the years (see Neto et al. 2010, for a complete description of the estuary's modifications) and both the river bed topography and the system hydrodynamics were altered, leading to the interruption of the communication between the two arms in the early 1990s (Marques et al., 1997; 2003; Neto et al., 2010), with severe impacts on the south arm. In this subsystem, the increase in water residence time and nutrient

concentration promoted eutrophication symptoms and the deterioration of the environmental quality (Marques et al., 2003). A gradual shift in primary producers from a community dominated by rooted macrophytes (*Zostera noltii*) to a community dominated by green macroalgae (mostly *Ulva* spp.) was observed (Marques et al., 2003), leading to a reduction in the *Zostera noltii* coverage area (Martins et al., 2005) and to a shift in benthic primary producers, affecting the structure and functioning of the biological communities (Marques et al., 1997; 2003; Martins et al., 2005; Patrício and Marques, 2006).

After the mitigation measures implemented to improve the system ecological condition in 1998 (the discharge of freshwater from the Pranto River decreased and the communication between the two arms was re-established) the system underwent partial improvements in its environmental quality (Teixeira et al., 2008; Cardoso et al., 2010), with a recovery of the *Zostera noltii* meadow and a cessation of the macroalgae blooms (Martins et al., 2005; Dolbeth et al., 2007; Patrício et al., 2009).

The recovery of the system allowed the identification of the high residence time as a cause for the ecological degradation in the south arm and suggested that the efficient renewal of water in this subsystem would increase the flow and load capacity of the water mass, which encouraged a complete re-establishment of the communication between both arms by the spring of 2006, decided at the Portuguese government level (Veríssimo et al., 2012a). The upstream connection between the two arms was enlarged and the hydraulic regime fully re-established (Veríssimo et al., 2012b). This investigation focuses on periods after the intervention.

Sampling strategy and laboratory procedures

Sampling was conducted during low tide on three occasions (September 2009, December 2009 and March 2010) in four intertidal areas of the south arm of the Mondego estuary, representing different environmental situations along a spatial gradient of eutrophication (Marques et al., 1997; 2003; Patrício and Marques, 2006) and with a gradient of coverage by *Zostera noltii*: a) a non-eutrophic area located downstream, where *Zostera noltii* predominates, and

considered the richest area of the estuary in terms of productivity and biodiversity (Marques et al., 1993; Dolbeth et al., 2007); b) an intermediate eutrophic area (*Zostera noltii* absent, although residual roots can be found and occasional formation of macroalgae mats observed); c) a bare sediment area in the inner part of the estuary where eutrophication processes occurred in the estuary (macrophyte community absent, regularly occurring blooms of *Ulva* spp.), currently characterized by a few, small and irregularly distributed *Z. noltii* patches (Veríssimo et al., 2013); and d) a bare sediment area located further upstream adjacent to the intervention area, with higher freshwater influence; hereafter referred as "Zostera", "Intermedia", "Armazens" and "Montante", respectively (Fig. 1).

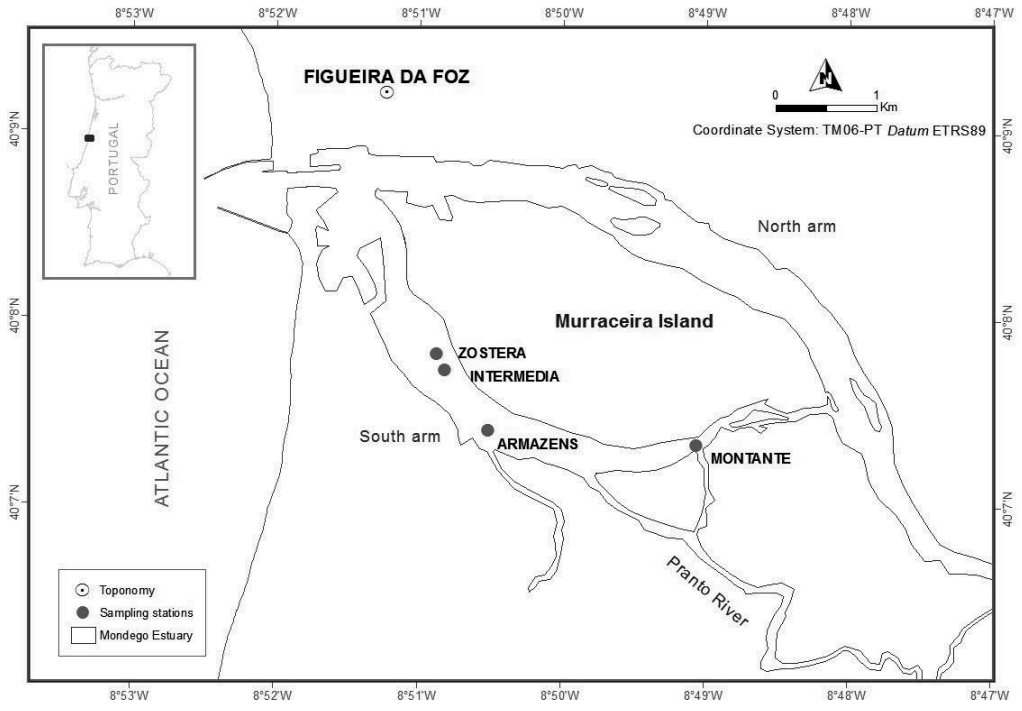

Figure 1. Mondego estuary. Location of the four intertidal sampling areas: "Zostera", "Intermedia", "Armazens" and "Montante".

Environmental variables

Bottom water variables were measured *in situ* at each area using an YSI Data Sonde Survey 4: salinity, pH, temperature (ºC) and dissolved oxygen (mgL^{-1}). Additionally, water samples were collected for laboratory determination of dissolved nutrients concentration: Nitrates (NO_3^--N) and nitrites (NO_2^--N) concentration (mgL^{-1}) were analyzed as described in Strickland and Parsons (1972) and ammonia (NH_4^+-N) and phosphates (PO_4^{3-}-P) concentration (mgL^{-1}) as described in the Limnologisk Metodik (1992). Sediment samples were taken to determine chlorophyll *a* concentration, organic matter content and grain size distribution. To obtain an approximate value for the microphytobenthos biomass, the top 1 cm of six 6.16 cm^2 replicates was sampled. The samples were carefully mixed, freeze-dried and kept in the dark at −20 ◦C until further processing. The Chl *a* concentration of the dried sediment was extracted in 90% acetone over 20 h in the dark; Chl *a* was then measured using a fluorometer, and expressed as g Chl *a* m^{-2}. The C:Chl *a* ratio was considered constant and equal to 40 mg C mg Chl a^{-1} (De Jonge, 1980) and carbon was converted to ash-free dry weight (AFDW) using the relation 1 g C = 0.45 g AFDW (Jørgensen et al., 1991).

Sediment organic matter (OM) content was estimated as the difference between the dry sediment (60 ºC for 72 h) and the sediment weight after combustion (450 ºC for 8 h), and expressed as a percentage of total sample weight. Grain size analysis was performed by dry mechanical sieving through a column of sieves of different mesh sizes and the Brown and McLachlan (1990) classification system was followed (gravel: >2 mm, coarse sand: 0.500–2.000 mm, medium sand: 0.250–0.500 mm, fine sand: 0.063–0.250 mm, and silt and clay: <0.063 mm). The grain size composition was expressed as the percentage of total sample weight.

Biological data: meiofauna and free-living nematodes

At each of the four areas, two sampling stations (A and B), separated by 20-30 m, were selected and three replicates were randomly collected at each station (covering a range of 10-15 m) in order to determine if patchy distribution was observed in meiofauna and nematode communities. Replicates were collected by forcing a sediment corer (inner diameter: 3.6 cm) 3 cm into the sediment and the

samples preserved in 4% buffered formaldehyde. To extract the meiofauna, the sediment replicates were sieved through 1 mm and 38 μm mesh size sieves and the fraction retained in the smaller mesh was centrifuged in Ludox HS-40 colloidal silica at a specific gravity of 1.18 g cm^{-3} (Vincx, 1996). The supernatant collected in the 38 μm mesh sieve was rinsed with water and stored in 4% buffered formaldehyde. Meiobenthic organisms were identified to major taxa level under a stereomicroscope following Higgins and Thiel (1988) and Giere (2009) and the density (individuals per 10 cm^2) of each taxon was computed. For nematode identification, a random set of 120 nematodes (if present), from each replicate were picked, cleared in glycerol–ethanol solution, transferred to anhydrous glycerol by evaporation and mounted on permanent glycerin slides for identification (Vincx, 1996). All nematodes were identified to genus level using a microscope fitted with a 100x oil immersion objective and the keys of Platt and Warwick (1983; 1988), Warwick et al. (1998), and the online information system NeMys (Steyaert et al., 2005). All identified individuals were grouped into four feeding-type groups (selective deposit feeders (1A), non-selective deposit feeders (1B), epigrowth feeders (2A), and predators/omnivores (2B)) according to the Wieser classification (1953). Furthermore, nematode genera were assigned a value on a colonizer-persister (c-p) scale accordingly their ability for colonizing or persisting in a certain habitat, in a continuum from "colonizers" (c; organisms with a high tolerance to disturbance events) to "persisters" (p; low tolerance) (Bongers et al., 1991).

Data analysis

Environmental variables

Environmental variables were analyzed through Principal Components Analysis (PCA) to search for potential spatial and temporal patterns. Prior to the calculation of the environmental parameters resemblance matrix using the Euclidean distance coefficient, the redundant variables were removed from the analysis so that the first two axes accounted for the maximum variability in the dataset. The variables retained in the model (organic matter, salinity, ammonia,

nitrate, phosphate, chlorophyll *a*, dissolved oxygen, silt+clay, coarse sand and gravel) act as proxy for the ones that were eliminated (pH, silicates, nitrite, fine sand, mean sand and temperature). Variables were square-root transformed (except dissolved oxygen) and then normalized.

Meiofauna and nematode communities

Biological data were analyzed in order to test for differences in meiofauna and nematode communities among sampling occasions and areas, both considering univariate and multivariate measures, through a series of permutational multivariate analyses of variance (PERMANOVA) using the PRIMER v6 software package (Clarke and Gorley, 2006) with the PERMANOVA add-on package (Anderson et al., 2008).

Preliminary one-way PERMANOVA analysis were performed to check for differences (patchy distribution) in meiofauna and nematode communities between stations A and B from each Area. As no significant differences were observed within each Area, data from both stations were pooled and the biological data were analysed considering six replicates in each Area.

All PERMANOVA analyses were performed using a two-way crossed design with two factors: Area (fixed, four levels: "Zostera", "Intermedia", "Armazens" and "Montante") and Sampling occasion (fixed, three levels: September 2009, December 2009 and March 2010). The 'Permutation of residuals under a reduced model' option was selected and 9999 permutations carried out. When significant differences ($p<0.05$) were detected, these were further examined using *a posteriori* pairwise comparisons. Euclidean distance similarity matrices were used for univariate data (meiofauna total mean density, meiofauna total number of taxa, nematode total density, genera diversity, Margalef index and Shannon-Wiener index) while the analysis of multivariate structure were conducted on Bray-Curtis similarity matrices, after square root transformed data (meiofauna composition, nematode genera composition). Total number of taxa and total mean density of individual major meiofauna taxa and of total meiofauna (individuals per $10cm^2$) were calculated for each area and sampling occasion. To visualize the multivariate data, a Principal Coordinates analysis (PCO) plot was drawn.

Free-living nematodes, the dominant taxon, were studied in particular depth. Besides the described two-way PERMANOVAs to test if nematode communities change spatially and temporally, the Index of Trophic Diversity (ITD) (Heip et al., 1985) was calculated as $ITD = \sum \theta^2$ where θ is the density contribution of each trophic group to total nematode density, ranging from 0.25 (highest trophic diversity) to 1.0 (lowest trophic diversity). Both the Index of Trophic Diversity and the trophic composition of nematodes community were analyzed through PERMANOVA analysis based on Euclidean and Bray-Curtis similarity measures, respectively, and following a similar design of the one described above. Furthermore, the Maturity Index (MI) (Bongers, 1990; Bongers et al., 1991) was calculated to analyze changes in the nematode's life strategy. Based on a colonizer-persister scale, the MI was calculated as the weighted average of the individual colonizer-persistent (c-p) values as $M = \sum_{i=1}^{n} v(i) f(i)$, where $v(i)$ is the c-p value of the taxon *i* and $f(i)$ is the frequency of that taxon. The contribution of each life-history group (c-p 1–5) to the total nematode assemblage was then calculated and, similarly to the described above, PERMANOVA analysis were performed for both Maturity Index and c-p classes composition using Euclidean and Bray-Curtis similarity measures, respectively.

To visualize the multivariate data, a Principal Coordinates analysis (PCO) plot was drawn. Afterwards, to determine the relative contribution of each genus to the (dis)similarities between sampling occasions and areas, a two-way crossed similarity percentage analysis procedure (SIMPER; cut-off percentage: 70%) was performed.

Relation between nematode assemblages and environmental variables

To assess to what extent environmental variables influenced the distribution of the nematode communities, a DISTLM (distance-based linear model) routine was applied. This routine is used for analyzing and modelling the relationship between a multivariate data cloud and one or more predictor variables, through the building of parsimonious models of variables that explain

the nematode genera community patterns. Environmental variables were first analyzed for co-linearity (redundant variables were removed and the ones kept act as proxy for the removed ones) and the following ten variables were used: silt+clay, coarse sand, gravel, organic matter, Chl *a*, salinity, dissolved oxygen, ammonia, nitrates and phosphates. DISTLM procedure was performed by forward selection of the environmental variables, using the R^2 as the selection criterion for fitting the best explanatory variables in the model, and 9999 permutations. This allowed also for the performance of marginal tests (individual variable relation with genera-derived multivariate data and significance level) (Anderson et al., 2008). To visualize the proposed model, a distance-based redundancy analysis (dbRDA) was done, resulting in a constrained ordination plot with axes linearly related to the fitted values and the predictor variables.

RESULTS

Environmental variables

The results of the PCA ordination (the first two PC axes accounted for 59.0% of the variability of the data) showed a separation of sampling stations according to the sampling occasion (with a clear separation of samples from March 2010 from the other two occasions) and according to their location along the south arm, where two groups were observed: 1) areas “Intermedia” and “Armazens” and 2) areas “Zostera” and “Montante”, presenting each group a similar environmental characterization (Fig. 2). During March 2010, higher concentrations of water nitrates and dissolved oxygen values were observed, while in September 2009 and December 2009, higher salinity, phosphates concentration and coarser sediments were observed. Regarding the differences between Areas, “Intermedia” and “Armazens” were characterized by higher chlorophyll *a* concentrations, lower amount of ammonia and silt+clay, while at “Montante” and “Zostera” areas higher amount of coarse sand and silt+clay granulometric classes prevailed, as well as higher concentration of organic matter (Fig. 2).

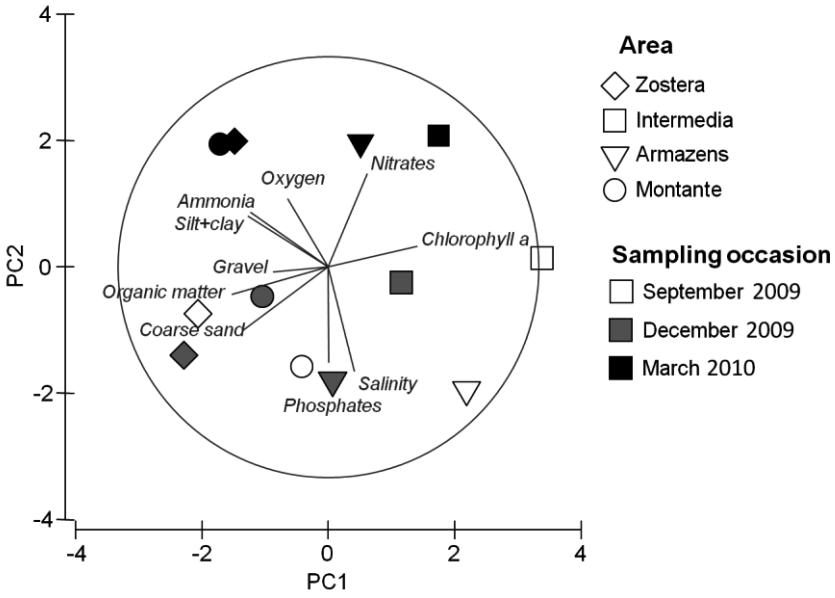

Figure 2. Principal Component Analysis (PCA) plot based on the environmental variables measured in each "Sampling occasion" (September 2009, December 2009 and March 2010) and "Area" ("Zostera", "Intermedia", "Armazens" and "Montante"). PC1 = 33.4%, PC2 = 25.6%.

Meiofauna communities

In total, thirteen meiofauna taxa were identified. Nematoda was always the dominant taxon (62.5-95.8%), followed by Polychaeta (1.0-29.4%) and Harpacticoid copepods (1.7-22.5%). Total meiofauna density ranged from 104.94±30.34 ind.10 cm^{-2} ("Zostera", March2010) to 2002.46±1248.70 ind.10 cm^{-2} ("Zostera", December 2009), and the number of taxa varied from 5 ("Zostera", March 2010) to 13 ("Armazens", December 2009). The results of the univariate PERMANOVA analysis of density indicated a highly significance for the interaction of the factors "sampling occasion" and "area" ($p<0.05$, Table 1A), with generally higher meiofauna density in December 2009, although this temporal trend was not consistent across all areas. Regarding the taxa number, significant differences existed between "sampling occasions" (December 2009>September 2009>March 2010) and "areas" (with "Armazens" presenting a higher taxa number than the remaining areas) (Table 1A).

The meiofauna community-based Principal Coordinates plot (Fig. 3) showed a clear separation of "sampling occasions", while a separation of "areas" was not so evident. PERMANOVA tests performed on meiofauna composition data

supported the observed patterns, with a significant interaction between the two factors. In all areas, significant differences existed among sampling occasions (except between September 2009 and March 2010 at "Armazens"), while a common pattern of differences between "Zostera" and "Armazens" and between "Armazens" and "Montante" was observed across sampling occasions (Table 1A).

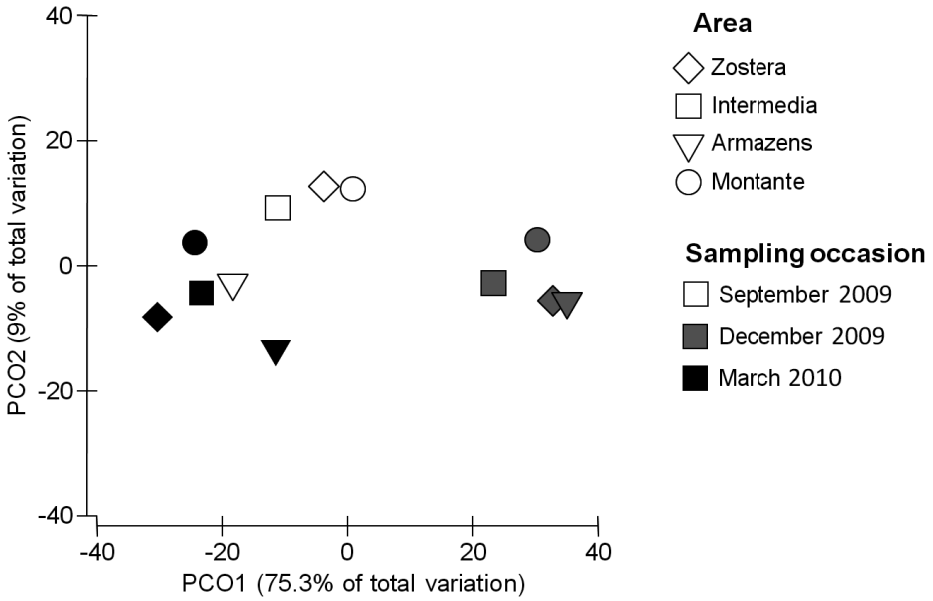

Figure 3. Principal Coordinates Ordination plot based on the meiofauna composition, in each "Sampling occasion" (September 2009, December 2009 and March 2010) and "Area" ("Zostera", "Intermedia", "Armazens" and "Montante").

Nematoda communities

Density and diversity

Nematodes dominated the meiofauna community, accounting between 62.5% ("Armazens", December 2009) and 95.8% ("Zostera", September 2009) of meiofauna density. The density of nematodes ranged from 90.86±25.11 ind.10cm^{-2} to 1746.89±1225.26 ind.10 cm^{-2}, both at the "Zostera" area (in March 2010 and December 2009, respectively), and a significant interaction between "area" and "sampling occasion" was observed regarding this parameter (PERMANOVA $p<0.05$), with a general pattern of lower density in March 2010 and higher density

in December 2009 across areas, while no regular pattern was observed across sampling occasions (Table 1B).

The community was composed by 46 nematode genera, belonging to 17 families. The dominant genera were *Sabatieria*, *Daptonema*, *Sphaerolaimus*, *Ptycholaimellus*, *Viscosia*, *Paralinhomoeus*, *Dichromadora*, *Terschellingia and Metachromadora*, accounting for about 81% of the nematode assemblages during the study period, with the remaining genera accounting for less than 2.6% (Table 2). The number of different genera ranged between 15 ("Montante", September 2009) and 37 ("Armazens", December 2009). PERMANOVA analysis revealed significant differences between areas and between sampling occasions (Table 1B), with higher genera number in areas "Intermedia" and "Armazens" and in December 2009.

The diversity indices (Margalef and Shannon-Wiener) broadly followed the patterns observed by the number of genera, with differences between all pairs of areas (except between "Zostera"-"Montante" and "Intermedia"-"Armazens", for Margalef index; and between "Intermedia"-"Armazens", for Shannon-Wiener index), and between sampling occasions (except between September 2009-March 2010 for both indices).

Community structure

Regarding the composition of nematodes a significant interaction between the factors "area" and "sampling occasion" was observed, with differences between all pairs of sampling occasions within each area and between each area pair across all sampling occasions (Table 1B). In agreement, the community-based PCO ordination plot (Fig. 4) shows a clear separation of samples accordingly the sampling occasions and, to a less extent, a separation of areas "Intermedia" and "Armazens" from areas "Zostera" and "Montante" areas can be also considered.

Table 1. Two-way PERMANOVA results of the comparison of the univariate and multivariate descriptors of the meiofauna (A) and nematode (B) communities, at each sampling occasion and area. Values in bold were significant at $p < 0.05$.

A. Meiofauna						
Density	**Source**	**df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**
	Sampling occasion	2	5414.7	2707.4	60.145	**0.0001**
	Area	3	398.64	132.88	2.952	**0.0373**
	Sampling occasion x Area	6	867.95	144.66	3.2137	**0.0077**
	Res	58	2610.8	45.014		
	Total	69	9274.8			
Number of taxa	Sampling occasion	2	326.12	163.06	127.29	**0.0001**
	Area	3	24.057	8.019	6.2597	**0.0011**
	Sampling occasion x Area	6	10.113	1.6855	1.3157	0.2609
	Res	58	74.3	1.281		
	Total	69	435.27			
Meiofauna composition	Sampling occasion	2	34366	17183	55 872	**0.0001**
	Area	3	5058.9	1686.3	5.4832	**0.0001**
	Sampling occasion x Area	6	4928.6	821.43	2 671	**0.0003**
	Res	58	17837	307.54		
	Total	69	62298			
B. Nematodes						
Total density	Sampling occasion	2	8420000	4210000	22.246	**0.0001**
	Area	3	2350000	785000	4.1429	**0.0075**
	Sampling occasion x Area	6	3580000	596000	3.1493	**0.0074**
	Res	58	11000000	189000		
	Total	69	25300000			
Number of genera	Sampling occasion	2	485.27	242.64	32.181	**0.0001**
	Area	3	275.23	91.744	12.168	**0.0001**
	Sampling occasion x Area	6	46.682	7.7803	1.0319	0.4171
	Res	58	437.3	7.5397		
	Total	69	1241.8			
Margalef Index	Sampling occasion	2	2.9763	1.4881	6.3588	**0.0034**
	Area	3	12.514	4.1714	17.824	**0.0001**
	Sampling occasion x Area	6	1.618	0.26966	1.1523	0.3405
	Res	58	13.574	0.23403		
	Total	69	30.391			
Shannon-Wiener Index	Sampling occasion	2	6.9259	3.4629	31.715	**0.0001**
	Area	3	5.2923	1.7641	16.156	**0.0001**
	Sampling occasion x Area	6	0.70081	0.1168	1.0697	0.3881
	Res	58	6.333	0.10919		
	Total	69	19.073			
Nematode composition	Sampling occasion	2	27184	13592	19.381	**0.0001**
	Area	3	15074	5024.7	7.1646	**0.0001**
	Sampling occasion x Area	6	10463	1743.9	2.4866	**0.0001**
	Res	58	40676	701.32		
	Total	69	93563			
Trophic composition	Sampling occasion	2	42071	21036	32.841	**0.0001**
	Area	3	8459.4	2819.8	4.4023	**0.0001**
	Sampling occasion x Area	6	10849	1808.2	2.823	**0.0001**
	Res	58	37150	640.53		
	Total	69	98402			

Table 1 continues in the next page

Table 1 (cont.)

ITD	Sampling occasion	2	0.15628	0.0781	13.035	**0.0001**
	Area	3	0.0519	0.0173	2.8832	**0.0403**
	Sampling occasion x Area	6	0.0245	0.00408	0.68002	0.6723
	Res	58	0.3477	0.00599		
	Total	69	0.57855			
MI	Sampling occasion	2	0.11809	0.059045	3.3794	**0.038**
	Area	3	0.63065	0.21022	12.032	**0.001**
	Sampling occasion x Area	6	0.14589	0.024316	1.3917	0.237
	Res	58	1.0134	0.017472		
	Total	69	1.9089			
c-p classes	Sampling occasion	2	18561	9280.6	43.465	**0.001**
	Area	3	2391.8	797.26	3.7339	**0.002**
	Sampling occasion x Area	6	3968.7	661.45	3.0979	**0.001**
	Res	58	12384	213.52		
	Total	69	37236			

The SIMPER analysis corroborated the pattern observed through the PCO analysis, showing higher dissimilarities between sampling occasions (September 2009 *vs* December 2009: 47.4%; September 2009 *vs* March 2010: 49.5%; December 2009 *vs* March 2010: 57.0%) than between areas (dissimilarities<47.7%). Regarding differences between Areas, the highest dissimilarity occurred between "Zostera" and "Armazens" (47.7%; mainly due to higher abundances of *Viscosia* and *Anoplostoma* at "Armazens" and *Sabatieria*, *Daptonema*, *Ptycholaimellus*, *Terschellingia*, *Dichromadora*, *Paralinhomoeus* and *Sphaerolaimus* at "Zostera"), while the lowest dissimilarity was observed between "Zostera" and "Montante" areas (42.1%) (Annex 6).

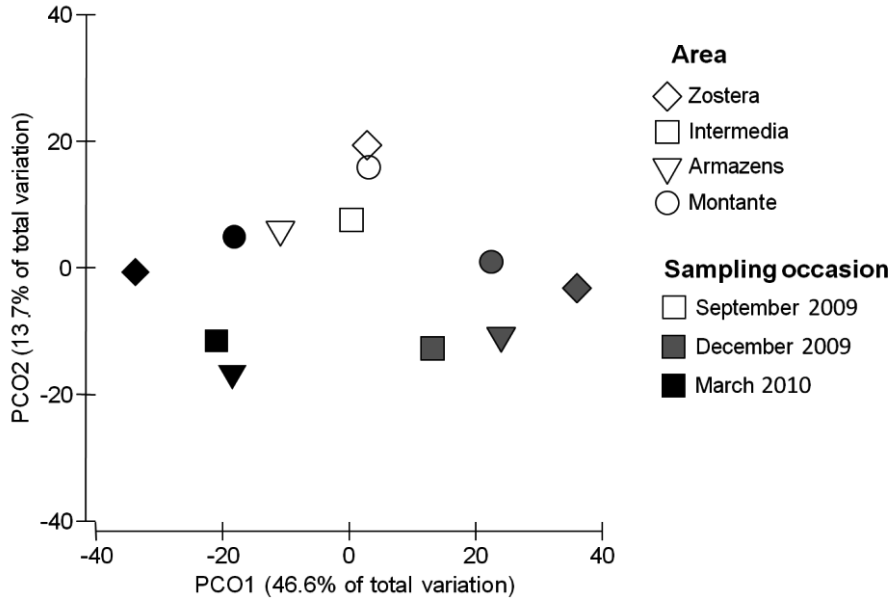

Figure 4. Principal Coordinates Ordination plot based on the nematodes genera composition, in each "Sampling occasion" (September 2009, December 2009 and March 2010) and "Area" ("Zostera", "Intermedia", "Armazens" and "Montante").

Trophic structure

The trophic composition revealed a community dominated by non-selective deposit feeders (1B: 52.2%) at all areas and sampling occasions (ranging from 37.6% at "Intermedia" in March 2010 to 69.1% at "Montante" in September 2009), followed by predators/omnivores (2B: 20.4%), epigrowth feeders (2A: 19.8%) and selective deposit feeders (1A: 7.6%). The variable distribution of feeding groups across areas and sampling occasions may explain the significant interaction in the PERMANOVA test (Table 1B, Fig. 5).

The Index of Trophic Diversity (ITD) ranged from 0.34±0.03 ("Intermedia", December 2009 and March 2010) to 0.52±0.09 ("Montante", September 2009). The index values presented significant differences among sampling occasions and among areas, with lower values in December 2009 (followed by March 2010 and September 2009) and with differences between "Intermedia" (lowest ITD value) and "Montante" (highest ITD value) areas (Table 1B, Fig. 5).

Table 2. Mean density (number of individuals 10 cm^{-2}) of nematode genera in each area ("Zostera", "Intermedia", "Armazens" and "Montante") and sampling occasion (September 2009, December 2009 and March 2010).

Genera	September 2009				December 2009				March 2010			
	Zostera	Intermedia	Armazens	Montante	Zostera	Intermedia	Armazens	Montante	Zostera	Intermedia	Armazens	Montante
Sabatieria	167.82	44.82	93.29	280.42	402.26	37.79	177.21	203.37	17.17	5.56	10.46	28.09
Daptonema	78.72	83.08	13.46	87.75	141.81	86.03	69.06	171.71	33.88	33.92	50.05	70.79
Sphaerolaimus	40.70	24.17	18.20	86.11	169.56	40.73	48.94	139.63	7.28	14.89	13.13	22.76
Ptycholaimellus	5.73	20.27	4.69	42.78	271.49	47.94	21.34	97.42		14.95	5.82	32.63
Viscosia	12.31	8.37	16.72	16.41	52.53	49.86	92.88	54.48	5.09	14.28	28.38	9.07
Dichromadora	23.79	13.88	5.76	12.45	96.6	30.87	20.63	11.54	17.21	28.13	5.37	0.98
Paralinhomoeus	26.84	23.13	4.00	20.67	65.67	22.73	47.96	36.68	2.77	2.19	0.99	2.94
Terschellingia	87.91	13.44	21.88	6.49	76.29	2.45	16.66	20.99	1.1	0.38	0.35	2.95
Metachromadora	0.56			4.11	66.12	16.19	9.6	73.98	2.02	0.79	1.12	10.51
Anoplostoma	8.68	6.78	8.51	10.24	67.09	6.53	21.75	9.86	0.19	2.43	13.87	3.62
Chromadora	1.56	9.41	0.20		61.99	16.86	13.18	14.31	0.49	1.27	0.18	0.58
Desmolaimus		0.79	0.39		74.45	3.06	15.93	1.97			0.46	
Microlaimus		1.27			67.00	9.37	14.06				1.4	0.18
Axonolaimus	2.67	3.89		1.40	18.63	14.83	15.63	7.79	0.19	7.88	1.2	0.5
Paracomesoma	22.44	41.02	0.89		0.79	5.24	2.29					
Linhomoeus	15.00	10.58	0.88	4.13	16.33	9.23	7.59		0.86	2.17	0.98	0.76
Leptolaimus		0.83			44.34	3.11		9.46		0.19		
Nemanema		0.80	2.00	2.70		3.63	34.97	1.02		2.38	2.39	0.67
Halalaimus		2.54	0.86		3.02	14.9	19.7	5.97	0.21			0.4
Metalinhomoeus	8.65	1.83	8.91		12.06	1.9	2.83	1.02	0.88	0.52	0.7	0.73
Calyptronema	1.67	14.09		1.40	1.4	4.5	9.24		0.65	3.91		1.12
Eleutherolaimus		3.12	0.81			9.23	20.83	2.76	0.19	0.48		
Odontophora			0.86		1.42	2.85	19.74	1.97		1.01	0.92	
Oxystomina			0.86		4.42	2.46	11.7	1.02	0.21	2.5	0.35	
Oncholaimellus		2.06			1.4	13.96	0.88			2.82	0.83	
Chromadorita			1.34		3.01	0.94	8.51	0.89			1.31	
Molgolaimus					4.48		6.93	0.94			2.06	

Table 2 continues in the next page

Table 2 (cont.)

Antomicron			0.39		2.82	2.41	3.72	1.83	0.21		1.36	
Paracyatholaimu	0.52		0.61		3.01	3.64	2.31		0.25	0.93	0.28	0.51
Prochromadorell		0.39	0.39				9.29	1.02				
Paracanthonchus					6.75	3.05						
Aegialoalaimus						1.9	1.45	1.68			0.64	
Camacolaimus					1.42		2.98	1.02				
Chromadorina			0.20		1.42		2.32				1.25	
Araeolaimus							4.55					
Oncholaimus					4.48							
Chromadorella							2.98					
Thalassoalaimus			0.59				2.31					
Cyatholaimus					2.83							
Spilophorella				2.70								
Cyartonema						0.95	1.41					
Theristus							0.88	0.89				0.29
Aponema		0.83										
Hypodontolaimus												0.8
Comesoma						0.61						
Bathylaimus			0.20									

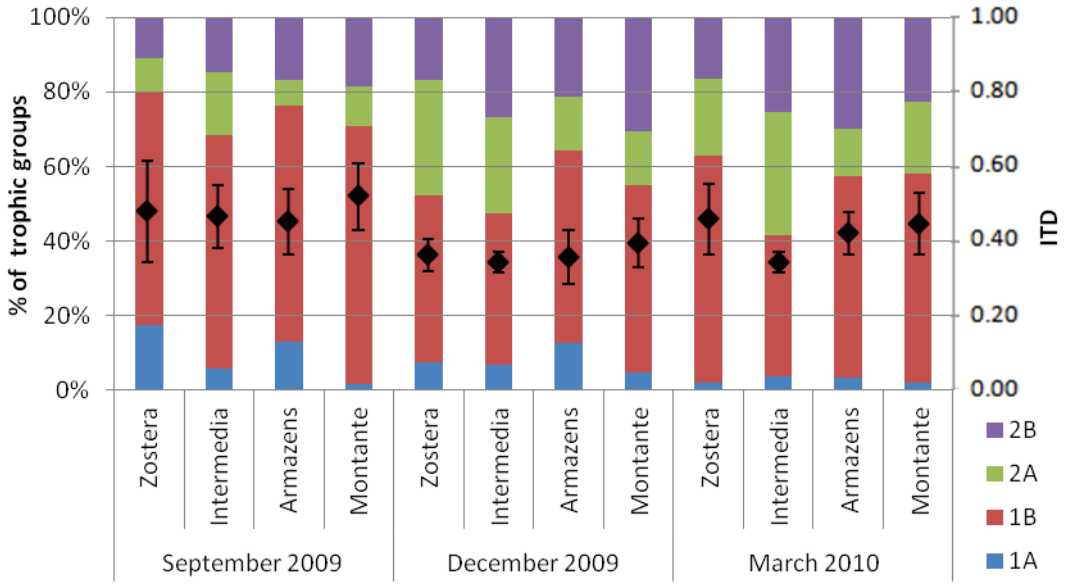

Figure 5. Percentage of contribution of the different trophic groups and Index of Trophic Diversity (ITD ± standard deviation) in each "Sampling occasion" (September 2009, December 2009 and March 2010) and "Area" ("Zostera", "Intermedia", "Armazens" and "Montante"). 1A – selective deposit feeders; 1B – non-selective deposit feeders; 2A – epigrowth feeders; 2B – omnivores/predators.

Life strategy structure

Most nematodes attained a colonizer-persister score of 2, ranging from 40.8% ("Intermedia", March 2010) to 79.5% ("Montante", September 2009), followed by c-p score of 3, ranging from 16.2% at "Montante", September 2009, to 55.5% at "Intermedia", March 2010. Persisters (c-p=4) were the least abundant, ranging from 2.4% ("Montante", March 2010) to 12.1% at "Armazéns", December 2009 (Fig. 6). However, the variable distribution of c-p classes across areas and sampling occasions resulted in a significant interaction between them being detected by the PERMANOVA test (Table 1B).

The Maturity Index ranged from 2.3 (at "Montante" in all sampling occasions and at "Zostera" March 2010) to 2.7 ("Intermedia", March 2010), with significant differences being observed among sampling occasions and areas (Table 1B, Fig. 6). In fact, higher MI values were observed in December, when compared to September 2009, while at "Montante" the MI was always lower, with differences also between "Zostera" and "Intermedia" areas (lower values at "Zostera") (Fig. 6).

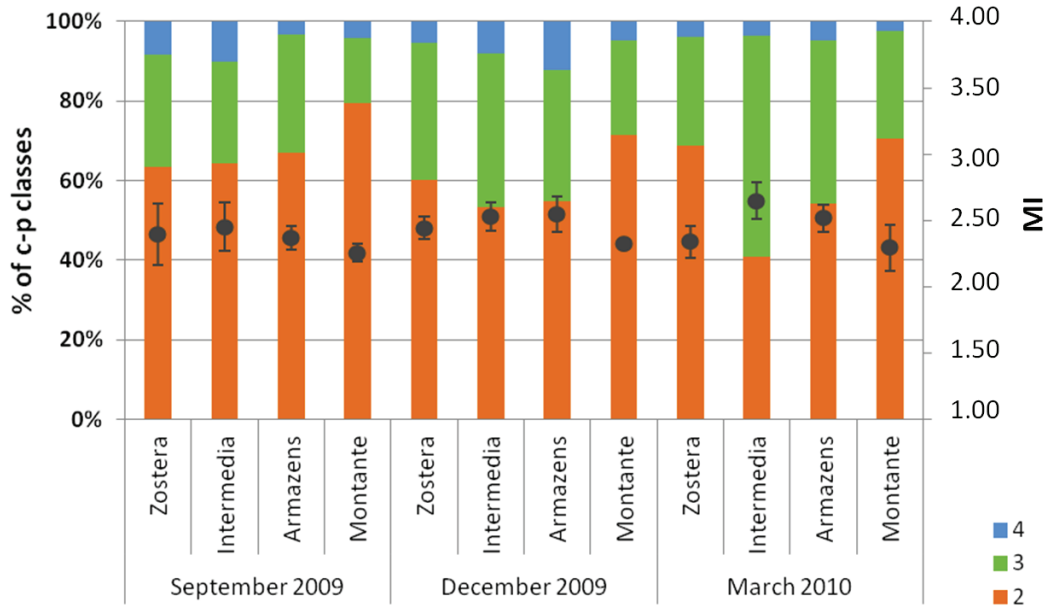

Figure 6. Percentage of contribution of the different c-p classes and Maturity Index (MI ± standard deviation) in each "Sampling occasion" (September 2009, December 2009 and March 2010) and "Area" ("Zostera", "Intermedia", "Armazens" and "Montante"). 2, c-p value=2; 3, c-p value=3; 4, cp value=4.

Relation between environmental parameters and nematode communities

Individual variables presenting a significant relationship with nematodes distribution pattern (marginal tests of the DISTLM, $p<0.05$) were phosphates ($p=0.0001$) and nitrates ($p=0.0271$), explaining alone nearly 42% and 23%, of the variation in the nematode genera composition, respectively. The best fitted model evidenced that a combination of four factors constituted the best explanatory model for the nematodes community pattern: phosphates, dissolved oxygen, ammonia and organic matter (cumulative % of explanation: 41.6%, 52.4%, 62.7% and 71.6%). These variables together explain 71.55% of the variation in community structure. After fitting these variables, the p-values associated with the conditional test to add the next two variables (Chl *a* and salinity) are not statistically significant ($p>0.16$). In fact, these variables were correlated with variables included in the model (organic matter and dissolved oxygen), adding thus little explanation to the model.

The dbRDA plot showed a pattern among samples suggesting gradients in the community structure that can be modeled by the variables included in the model. The first two dbRDA axes explain 78.14% of the fitted variation, and this is about 55.91% of the total variation in the resemblance matrix (Fig. 7). This plot shows a remarkably similar pattern to the PCO ordination plot, indicating that the four variables included in the model are indeed capturing the most salient overall patterns of variability.

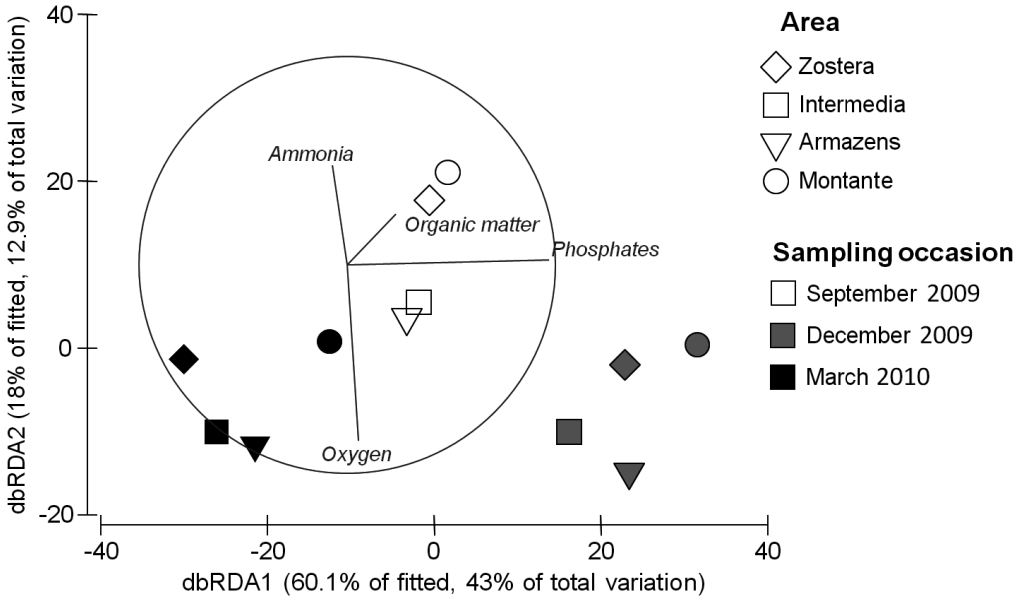

Figure 7. Distance-based redundancy (dbRDA) plot illustrating DISTLM model based on nematodes community and the fitted environmental variables as vectors (phosphates, dissolved oxygen, nitrates and organic matter).

DISCUSSION

The analyses of the intertidal meiobenthic communities of the Mondego estuary, with special emphasis on free-living nematodes, allowed filling the gap of knowledge regarding the distribution of these communities after the application of the mitigation measures implemented in May 2006**.** Several studies exist regarding other biological elements (zooplankton: Falcão et al., 2012; macrobenthic communities: Dolbeth et al., 2007; Cardoso et al., 2007; Veríssimo et al., 2012a; Marques et al., 2013), most of them comparing communities before and after the

intervention. A similar comparison cannot be provided for meiobenthic communities since no sampling was conducted prior to the spring of 2006 for meiobenthic communities. Regardless of that, the results obtained provide a general picture of the spatial distribution of meiofauna and nematodes in a restricted area of the estuary (maximum distance between areas ~3km), with historical modifications being known, and their temporal variation.

Environmental characterization of the South arm

The environmental characterization based on the PCA did not display an evident spatial segregation of the sampled areas, not following the estuarine gradient. Similar results were observed by Veríssimo et al. (2013) based on a similar sampling design. This evidence will have an important role in the interpretation of the meiobenthic communities distribution since potential differences regarding communities' features may not be easily ascribed to the natural estuarine gradient.

Seagrass beds are important in primary production, nutrient cycling and sediment and nutrient trapping (Orth et al., 2006; Fonseca et al., 2011). Since their presence reduces physical stress, it is not surprising that the "Zostera" area was characterized by the finest sediments and highest organic matter content, which is consistent with enhanced detritus deposition inside vegetated areas (Leduc and Probert, 2011). Other studies have observed sedimentary modifications caused by the presence of seagrass beds, compared to unvegetated areas (Fonseca et al., 2011), reinforcing the potential of seagrass beds as ecosystem engineers (Wright and Jones, 2006; Fonseca et al., 2011).

In spite of the spatial proximity of "Zostera" and "Intermedia" areas, higher similarities were observed between "Intermedia" and "Armazens" areas, mainly caused by the high chlorophyll *a* concentration and lower nutrients concentration, while the similarity between "Montante" and "Zostera" areas was induced by the higher content of fine sediments and organic matter.

In addition to the spatial variability, the temporal variation in the abiotic parameters (also observed by Baeta et al., 2009), with a more homogeneous physicochemical composition among the sampled areas in March 2010, can be

related with the climatic variations observed during the sampling period. The extreme climatic events felt in the area included a severe drought period from March to October 2009, followed by a period of heavy rain and flooding from November 2009 until April 2010 (Instituto de Meteorologia, IP, 2009a, 2009b, 2010), which might have been responsible for the reduced salinity values observed in March 2010, which in turn may have had repercussions in the meiobenthic features.

Meiofauna communities

The composition of the meiofauna communities was similar to that observed in the subtidal area of the Mondego estuary (Alves et al., 2013) and to other estuaries in intertidal areas (Smol et al., 1994; Soetaert et al., 1995; Rzeznik-Orignac et al., 2003; Bick and Arlt, 2005), with a dominance of nematodes, polychaetes and harpacticoid copepods. Nematodes' dominance is a common feature and is well documented (usually 60-90% of meiofauna communities are composed by nematodes; Coull, 1999). The second ranked taxon (polychaeta) only presented higher abundances than copepods in December 2009 and, in an overall analysis, this rank is altered if nauplii larvae stages are considered (and added to adult stages), with harpacticoid copepods ranking second, the most common pattern observed in estuaries (Coull, 1999).

Both nematodes and copepods (and most of the taxa) density peaked in December 2009 (autumn season), contradicting previous studies stating that, in temperate regions, meiobenthos are known to vary seasonally and usually peak in the warmest months (Smol et al., 1994). The decrease in abundance in the remaining seasons may be correlated with the extreme climatic events felt in the region. These events may have altered the salinity (lowering values from September 2009 to March 2010) and may have also caused sediment displacement and erosion, as well as changes in interstitial water salinity (Santos et al., 1996), thus affecting meiobenthos structure.

On average, a higher density of meiofauna (caused by high nematodes density) was encountered at the "Zostera" area, even though the differences encountered among sampling occasions, reinforcing the influence of the finer and

organically-rich sediments associated with seagrass meadows in enhancing nematodes density (Castel et al., 1989; Danovaro, 1996; Edgar, 1999; Danovaro et al., 2002; Leduc and Probert, 2011). Harpacticoid copepods also presented higher abundance at the "Zostera" area, and studies comparing abundance of copepods inside and outside seagrass beds have also found a higher density in the vegetated areas (Ansari and Parulekar, 1994; Guerrini et al., 1998; Ndaro and Olafsson, 1999; De Troch et al., 2001).

Regarding the taxa number, the maximum diversity found at "Armazens" may be related to the contribution of mean sand, which may have contributed for the creation of a wider range of microhabitats, with different niches being available for meiofauna elements (Smol et al., 1994). Furthermore, the meiobenthic ecosystem is also subjected to stochastic factors, such as local irregular and temporary disturbances and benefits (food input), contributing to the unpredictability of meiofauna distribution, even when alterations are of a small-scale nature (Giere, 2009). In fact, in spite of the pattern encountered in the abiotic environment along the south arm, meiofauna distribution did not closely follow it, and a clear temporal pattern was observed in meiofauna communities, overlapping the spatial one. Contrary to what was expected, meiofauna composition at the "Zostera" area was not different from the remaining ones. In Australia, Fonseca et al. (2011) compared meiofauna communities between vegetated and unvegetated sediments, concluding that, in contradiction to the findings of this study, discrete communities were observed, with little overlap in species composition.

Nematode communities

Nematode densities were within the range of density values from other intertidal studies (Smol et al., 1994; Soetaert et al., 1994; Steyaert et al., 2003). Comparing the intertidal density values with the ones from the subtidal zone of the Mondego estuary (Alves et al., 2013, limiting the comparison to the south arm), generally higher density values were found in the intertidal areas (similar findings were observed by Smol et al., 1994), which may be related with the high amount of finer sediments and organic matter in the intertidal area (Smol et al., 1994).

Even though no clear pattern regarding nematode density was observed, the highest values observed in December 2009, accompanied by the highest diversity measures (both number of genera and diversity indices), may indicate that the effect of temporal variations in nematode communities is important, at the analyzed spatial scale. In fact, Phillips and Fleeger (1985) have highlighted that temporal variations occur at a variety of spatial scales. Moreover, salinity is a factor controlling nematode distribution and, according to Ferrero et al. (2008), salinity range has a great impact of species distribution along estuaries, sometimes at a higher extent than sediment characteristics, reinforcing the role that the variable environmental conditions occurring in intertidal areas present in structuring nematode's composition and distribution.

At the spatial level, the highest density observed at "Zostera" area, together with a high diversity at "Intermedia" and "Armazens", indicates that different environmental factors are responsible for these features, with sediment granulometry exerting an important influence on the diversity of nematodes (Steyaert et al., 2003), with a wider variety of microhabitats being available at sandier sediments, enhancing diversity (Heip and Decraemaer, 1974).

Similarly to other estuaries, nematode communities comprised a high number of genera but with few dominant ones (Warwick, 1971; Austen et al., 1989; Li and Vincx, 1993; Soetaert et al., 1995; Rzeznik-Orignac et al., 2003; Steyaert et al., 2003; Ferrero et al.; 2008, Alves et al., 2013). In fact, the five most abundant genera (*Sabatieria*, *Ptycholaimellus*, *Daptonema*, *Sphaerolaimus* and *Paralinhomoeus*) accounted for a high percentage of density (56-82% and 62-75%, in each area and sampling occasion respectively), corroborating the dominance of fewer species in estuaries, as stated by Coull (1999).

Differences in geochemical and physical properties on a horizontal scale are known to be reflected not only in nematode abundance and diversity, but also in species composition and trophic structure (Steyaert et al., 2003). Regarding communities' multivariate structure, the seasonal effect seems to be superimposed to the spatial one, as also observed by Phillips and Fleeger (1985) and Smol et al. (1994), reinforcing that, in temperate regions, intertidal communities are known to vary seasonally (Smol et al., 1994). Also, nematode trophic composition revealed

a similar structure regardless of the area, with a dominance of non-selective deposit feeders. According to Bacelar-Nicolau et al. (2003) the bacterial dynamics in the south arm are mainly affected by temporal gradients, and less by the spatial structure, which can also be responsible for the distribution of nematodes, mainly feeding on bacteria.

The life strategy characterization and the widely used Maturity Index, which provide important additional informational to the one given by the trophic composition (Bongers et al., 1991) by relating the diverse strategies of nematodes to different disturbances, enabled a rough separation of sites, with the higher values in the inner stations being related to less disturbed conditions. On the other hand, both Zostera and Montante areas (expected to present opposite classification), revealed lower MI values. In fact, this differences were accounted for the higher abundance of colonizers (c-p=2) at these areas, while intermediate and persisters (c-p=3 and 4) were more abundant at the inner areas.

Besides the higher density in organically enriched and finer sediments, and higher diversity on sandier sediments, at this small spatial scale other environmental factors stood out as most responsible for the distribution pattern of nematode communities and the relationship between the abiotic environment and nematode communities highlighted the importance of dissolved oxygen, organic matter and water nutrients as structuring factors of the nematode communities.

Effectively, nematodes are affected by oxygen variations, and both field surveys and experimental work have reported their tolerance to oxygen deficiency, although densities are impaired (Neira et al., 2001; Levin, 2003; Steyaert et al., 2007). However, different tolerances were observed according to the species (Steyaert et al., 2003) indicating that nematode species are differentially adapted to living in or surviving in low oxygen environments. Regarding the influence of organic matter in nematodes distribution, the distribution of food availability, usable in different forms, affects the distribution and density of nematodes (Montagna, 1995; Moens et al., 1999). It is also interesting to scrutinize the influence of these factors in the perspective of the system's recovery, bearing in mind that the parameters chosen as the best to describe the biotic pattern also presented correlations with others, and so the importance of Chl *a* (the next

variable entering the model) must not be neglected. In fact, the environmental characterization may have influenced the trophic diversity along the south arm, which was highlighted through the Index of Trophic Diversity. This index, generally used to relate trophic diversity with pollution levels (Heip et al., 1985), revealed a better distributed community in December 2009, as well as at "Intermedia" area, while at "Montante" area a less diverse trophic community was observed, which may be related to the freshwater input felt in this area, being responsible for a different community structure and enhancing the presence of predators. Furthermore, the similarity regarding c-p composition and Maturity Index observed at Zostera and Montante may be resultant from opposite situations, since colonizers may occur both under food-rich (as at "Zostera") as well as food-poor conditions (as at "Montante") (Bongers and Bongers, 1998).

Past recovery and current status of the intertidal South arm stretch

Eutrophication is typically related to the increase of nutrient and organic matter loads, which can induce a progressive reduction in oxygen availability (Cloern, 2001), leading to hypoxia or anoxia. Therefore, sediments and benthic communities appear to be the most sensitive compartment to eutrophication and hypoxia (Jørgensen and Richardson, 1996). Meiofauna, due to their short life cycle, high turnover rates and lack of larval dispersion are expected to rapidly respond to environmental changes and food availability (Danovaro et al., 2002; Austen and Widdicombe, 2006; De Troch et al., 2006), and nematodes have been largely utilized as indicators of organic disturbances (Bongers and Ferris, 1999; Vanaverbeke et al., 2004), since they are known to persist and even increase their importance under long periods of hypoxic-anoxic conditions (Heip et al., 1985; Modig and Olafsson, 1998).

In the Mondego estuary, the analysis of the system's recuperation has favoured the response of macrobenthic communities towards restoration (Veríssimo et al., 2012a; Veríssimo et al., 2012b). However, meiofauna communities can also give important insights regarding pollution monitoring programs, complementing macrofauna's information, due to different "response-to-stress" time of each benthic group (Patrício et al., 2012), and while nematode

communities have increasingly been used to assess the effects of environmental perturbations (e.g. Gyedu-Ababio et al., 1999; Guo et al., 2001), few studies have focused on their recovery response to organic pollution (Liu et al., 2011). However, although the relation of nematodes and anthropogenic pressures in estuaries are somehow known, there is a difficulty in ascribing the individualization of the impacts, since not only different types of perturbations may occur simultaneously (Moreno et al., 2008), but also the environmental conditions in these areas are highly variable (Dauvin, 2007; Elliott and Quintino, 2007).

The observed patterns of density and diversity in the south arm of the Mondego estuary seem to be typical of many estuaries, not presenting strong evidence that severe impacts, due to the system's eutrophication history, persist at present. Similar evidences were found in the Thames estuary, following a long history of anthropogenic impact and recovery (Ferrero et al., 2008). In fact, in the Thames estuary, the comparison of nematode communities after a severe impact of pollution suggested that although differences were observed, the actual community resemble those of other European estuaries, indicating that some degree of recovery and re-colonization has taken place, parallel to the reduction of the pollution levels (Ferrero et al., 2008).

The distribution of the nematode communities in the studied area was expected not only to follow the eutrophication gradient, with a reduction in diversity and density of meiofaunal communities towards the inner part of the estuary, but also to present differences between the "Zostera" area and the remaining ones. Usually, habitats with the presence of seagrass are expected to be more diverse than those where it is absent (e.g. Boström and Bonsdorff, 1997; Connolly, 1997; Fredriksen et al., 2010), and studies comparing meiofauna communities from seagrass beds and unvegetated sediment (Tietjen, 1969; Alongi, 1987; Ndaro and Ólafsson, 1999; Fisher and Sheaves, 2003) have noticed that meiofauna is more abundant and diverse in seagrass beds (Alongi, 1987; Fisher and Sheaves, 2003, Fonseca et al., 2011).

However, the absence of structural differences in the nematode's communities could be explained by the physical and chemical processes that the estuary suffered from and that, at a certain moment, may have induced the

disruption of the communities. When the conditions became favourable (after the implementation of mitigation measures), a colonization of the sediment occurred along the entire arm. Dominant genera across the subsystem were similar (*Terschellingia*, *Sabatieria* and *Daptonema*) and are known to withstand harsh conditions, being typical of poorly oxygenated and organically enriched bottoms around the world (Soetaert et al., 1994; 1995; Schratzberger et al., 2007; Steyaert et al., 2007; Armenteros et al., 2009). One may hypothesize that, during the impacts, only the most resilient genera have survived and withstand the variable conditions, and a posterior colonization may have had taken place. According to Ferrero et al. (2008), re-colonization from within the estuary is able to happen: during the pollution impact sufficient refugia may exist for nematodes to re-colonize relatively quickly by transport in the water column. Furthermore, the impact on infaunal function due to seagrass effect on sediment characteristics and organic matter input (Leduc and Probert, 2011) was not observed since the trophic structure of the community was no variable along the south arm, indicating that this stretch behaves like a coherent subsystem recovering from the pressures suffered in the past.

CONCLUSIONS

To best of our knowledge this was the first attempt to analyse meiobenthic and nematode communities in an intertidal area that has suffered from eutrophication pressures in the past and where an eutrophication gradient could be followed. Since no data from before the implementation of the mitigation measure are available regarding meiobenthic communities, no before-after comparison was possible. However, the response of intertidal meiobenthic communities (both structure and function) revealed that, superimposed to the spatial gradient, the temporal effect seemed to be more relevant for the distribution patterns of the intertidal communities and the absence of evident differences between areas may indicate that the system has recovered from the early situations and a database for future comparisons becomes available.

General discussion

GENERAL DISCUSSION

> "Meiofauna are not impressively large or tasty, and they are not even dangerous – they are simply small. Meiofauna, organisms beyond our normal range of perception, are therefore intuitively uninteresting to most people, even to some in the scientific community, despite the productive capacity, ecological adaptability and environmental sensitivity of these tiny creatures."
>
> Giere, 2009

1. Meiobenthic communities in the Mondego estuary: what triggered their study?

The present work was focused on the meiobenthic communities of the Mondego estuary (Portugal), a South-Western European transitional system that suffered intense anthropogenic pressure over the last decades, with known overall decline in its environmental quality. A description of the alterations the estuary suffered from was performed along the Chapters and is summarized by Neto et al. (2010). The system's evolution and condition has been followed in the scope of both research projects and monitoring programs, with special emphasis on water quality, hydraulics, sediment dynamic and biological communities. Regarding benthic communities, a large dataset exists for macrobenthic invertebrates, with available information from before and after the mitigation measure that took place in Spring 2006, allowing investigating the response of the ecosystem to a new situation (e.g. Patrício and Marques, 2006; Patrício et al., 2009; Cardoso et al., 2010; Neto et al., 2010; Baeta et al., 2011; Dolbeth et al., 2011). Concerning meiobenthic communities, no similar database exists, hindering similar approaches to be performed. However, meiobenthic investigation has been enhanced and recent research projects performed in the estuary allowed the collection of both macrobenthic and meiobenthic samples, covering also several elements of water and sediment quality, allowing to start a database of meiobenthic and nematode communities.

In this scope, meiofauna investigation in the Mondego estuary benefited from the approval and performance of two distinct scientific projects ("EFICAS", POCI/MAR/61324/2004 and "RECONNECT", PTDC/MAR/64627/2006) which *i*) proposed some methods to assess the effects of freshwater discharges and associated salinity decrease on the benthic communities of two Portuguese estuaries (Mondego and Mira), with different anthropogenic impacts (results presented in Chapters 1 to 3), and *ii*) intended to study the system response to the total re-establishment of the upstream connection between the two arms of the Mondego estuary, with the associated implications for recovery and system's management (results in Chapter 4).

This allowed not only to sample meiofauna on a regular basis creating a dataset that is of value to follow the communities, both in intertidal and subtidal habitats, but also to determine the main factors structuring meiobenthos and nematode in estuarine systems, creating conditions for their coherent analysis and leading to the development of the works presented herein.

2. Meiobenthic communities in the assessment of estuarine ecological conditions

The complexity of meiobenthic distribution in estuaries was tackled, aiming at achieving a good data structure to allow disentangling the factors driving the observed patterns. By analyzing different habitats (subtidal and intertidal), the spatial and temporal distribution of meiobenthic and nematode communities was analyzed by different methodological approaches, including multivariate methods, hypothesis testing methods, different types of ecological indicators based on diversity and on ecological strategies, and single and multi-trait approaches (Biological Trait Analysis). These studies allowed answering the questions initially posed and raised new ones that are of extreme interest, not only because they are novelties regarding nematode communities but also for the applicability of their outcomes, which have only been explored at a theoretical level (see section 3 and 4 below). Furthermore, the different approaches allowed the assessment of diverse features of the estuarine system.

The distribution constraints, ecological and functional characteristics of meiobenthic and nematode communities were determined, followed by the identification of nematode key features to assess environmental status in estuaries. Along this thesis different patterns arose when studying the system at different spatial and temporal scales, which are worth to explore.

2.1. Spatial distribution: the estuarine gradient

The analysis of the subtidal meiobenthic communities at a major taxa level (Chapters 1 and 2) allowed the determination of their composition, which was similar to what is found in other European estuaries (e.g. Li and Vincx, 1993; Soetaert et al., 1994; 1995). Meiobenthic communities are mainly composed by nematodes, polychaetes and copepods, and their distribution pattern shows a gradient that is closely linked with the estuarine gradient (Patrício et al., 2012; Alves et al., 2013).

By increasing the taxonomic resolution, with the investigation of nematode genera distribution (Chapters 1 and 2) it became clearer that nematodes are the ones that best mirror the estuarine gradient, with different communities characterizing different predefined sections of the estuary (Teixeira et al., 2008). In fact, when comparing the "pictures" of the estuary provided by the analysis of the macrofauna and nematodes communities, a clearer pattern of separation of the areas arose regarding the nematode communities, confirming the separation of the estuarine areas based on an environmental characterization.

Although the comparative approach regarding macrofauna and nematode communities was only performed on a short temporal range (one season), it allowed highlighting that the diverse life histories of these communities integrate differently the environmental constraints, being recommended that both groups should be used in pollution monitoring groups, since they may integrate different aspects of the system, revealing complementary aspects of the factors structuring the benthic ecosystem (Vanaverbeke et al., 2011; Patrício et al., 2012).

In Chapter 2, besides describing the distribution patterns of density and diversity, that closely followed the estuarine gradient, maturity and trophic diversity indices were applied, presenting some opposite trends. This allowed the

identification of some knowledge gaps regarding their useful application, leading to new questions to be raised (see section 3 and 4 below). Nevertheless, the application of the referred indices enabled the recognition that different areas of the estuary present different constraints to the structure of the communities and, when assessing their ecological status, different functional aspects must be taken in consideration.

Moreover, based on the functional structure of the communities, it was possible to further recognize that this estuarine division is not only based on environmental characteristics but also on ecological ones, reinforcing the utility of functional analysis. It is recognized that changes in biodiversity may modify ecosystem function (Hooper et al., 2005) and taxonomic analyses may omit key functional aspects (Frid et al., 2000; Bremner et al., 2003), being recommended the inclusion of functional properties in the assessment of environmental change (de Jonge et al., 2006).

Along Chapter 3, the detailed analysis of biological traits presented by nematodes allowed, on one hand, to reinforce the knowledge on their distribution patterns along the estuarine gradient, understanding the effect of the most structuring variables and, on the other hand, enabled to determine that different insights on the system were highlighted by single and multi-trait analysis. Single traits analysis was, in fact, especially competent in disentangling the effects of abiotic estuarine variability, reinforcing their potential role as indicators of different environmental conditions (Tita et al., 1999; Soetaert et al., 2002; Vanaverbeke et al., 2004; Moreno et al., 2011). The work presented in Chapter 3 also reinforced the findings of Schratzberger et al. (2007) by verifying a similarity in the distribution of single and multi-traits along the estuary. Nevertheless, there is never an overlap of the information, demonstrating that the inclusion of diverse aspects of the functioning of the system allows a more realistic image of the systems to be obtained. Furthermore, it was also illustrated that information regarding biological traits is scarce for nematodes and even the basis of the Maturity Index and Index of Trophic Diversity rely on information that may not be the most accurate. This has been highlighted by Moens et al. (2005), Schratzberger et al. (2006), Schratzberger et al. (2008) and Moreno et al. (2011), encouraging

new information on traits to be acquired. In order to improve it, studies regarding trophic analysis with the application of stable isotopes and based on microcosm experiments would be beneficial for the correct determination of the trophic guild of each genus (Moens et al., 2005; Schratzberger et al., 2008). Furthermore, the correct assignment of marine genera to a colonizer-persister scale based on empirical support would also be useful (Schratzberger et al., 2006) (see section 3 and 4 below). Consequently, obtaining a greater knowledge of the functional roles of nematode species will be the key to improve the sensitivity and interpretation of biological traits analyses of benthic communities.

2.2. Temporal distribution: the effects of time and climate events

The dataset gathered for this thesis is in itself a valuable contribution as for the first time a temporal series of meiobenthos and nematodes was gathered for the Mondego estuary. Even if considered short, comparatively to the database of other benthic components, this database allowed to understand how communities are distributed along the estuary and how they vary along the year and when facing extreme climate events.

When analyzing the variability at a lower spatial scale, like in the work presented in Chapter 4, where meiofauna and nematodes at the South arm of the Mondego estuary are analyzed, a different pattern from the one presented in Chapters 1 to 3 was observed. By taking a small scale approach, focusing only on the polyhaline stretch, temporal differences were observed, differently from the larger scale (whole estuary) studies previously presented.

Extreme climatic events also play an important role in the structure of the communities and, although unpredictable, droughts and floods are known to influence meiobenthos and nematode communities, causing salinity alterations and sediment disruption (Santos et al., 1996; Ferrero et al., 2008). In this regard, however, the climatic event of severe flood during the Autumn 2006 (http://snirh.apambiente.pt/) had effects over the environmental characterization of the estuary, with consequent variations in the spatial distribution of meiofauna and nematodes, related to the referred salinity variations. Furthermore, extreme climatic events were also reported from March to October 2009 (drought) which

even if not affecting the subtidal communities, may have modified the intertidal ones, as well as the heavy rain from November 2009 to April 2010 (Instituto de Meteorologia, IP, 2009a, 2009b, 2010). These events may have forced an homogenization of the communities leading to a not so clear separation of the estuarine zones when they occurred, hampering also the identification of spatial assemblages differences at a smaller scale.

The described distribution patterns and related factors allowed to not only detect trends in meiobenthic distribution but also to highlight factors that must be concerned in environmental assessments. From a management perspective, it is first needed to know the distribution trends of the communities and their structuring factors to correctly analyze the effects of anthropogenic impacts. In fact, if physicochemical conditions are altered, these will have impacts on the structure of the communities, which, in turn, may affect higher trophic levels, which should be considered when applying well structured assessment actions. The complementarity between taxonomic and functional approaches allowed for a better knowledge of the system, which may have future implication in assessing different areas of the estuary known to present discrete communities. This allowed also to recognize that the application of tools to assess the system's ecological status should be performed with caution. In fact, it is suggested that the interpretation of the applied indices (ITD and MI) would benefit from more accurate information and from adjustment in the indices boundaries, aiming at correctly distinguish natural and human-made impacts.

Based on the knowledge gained along this thesis a further step towards a nematode-based multimetric index for assessing the ecological condition of estuarine systems became imperious. Since this theme is of interest and its development would be highly recommended, a detailed description was inserted in this Discussion section.

3. The integration of meiobenthic communities in the assessment of ecological quality status: next steps towards their inclusion in European Directives

Ecologists attempt to make predictions about the effects of environmental stressors on the structure, function and stability of aquatic food webs. Being fundamental elements of the trophic webs, meiobenthos elements have an important role in energy transfer to higher levels, and their assessment, parallel to the assessment of other biological communities or individually, should be the next step.

The works presented in this thesis allowed recognizing that there is enough ground information to pursue further objectives. In fact, as referred in Chapters 1 and 2, there is the need of a multimetric index regarding nematode communities. This would-be a major step in meiobenthic studies.

Over the last years, the implementation of the European Water Framework Directive (WFD, Directive 2000/60/EC) and the Marine Strategy Framework Directive (MSFD, Directive 2008/56/EC), reinforced the role of the biological elements as good indicators to assess environmental quality, since they integrate both the biotic and the abiotic components of an ecosystem through their adaptive responses (Casazza et al., 2002). The requirement of the European policies on the use of well-founded ecological indicators stimulated the development of this research field and they have become a popular tool for ecological assessment in aquatic ecosystems. Over the last decades, several assessment tools using macroinvertebrates in particular have thus been proposed by the scientific community (e.g. Pinto et al., 2009; Hering et al., 2010).

To date, however, few nematode-based indices are available for assessing the ecological condition of estuarine systems (Moreno et al., 2011) and multimetric indices in particular, are rather demanding. Studies relating nematode communities to system's environmental quality status have mostly applied the indices of Trophic Diversity (ITD, Heip et al., 1985) and Maturity Index (MI, Bongers, 1990; Bongers et al., 1991), which are based on feeding type (based on buccal cavity) and on life strategies, respectively. Although these indices have

shown potential to distinguish polluted from unpolluted sites (Heip et al., 1985; Essink and Keidel, 1998; Mirto et al., 2002; Moreno et al., 2008), their power to detect subtle changes is not exempted of criticism, since, for instance, if confounding factors such as differences in water depth, grain size, salinity fluctuations and food sources exist, which affect nematode abundance and distribution (Essink and Keidel, 1998; Moreno et al., 2008), the indices may not be able to detect other pressures.

There are other indices that are based on nematode indicator species (based on sensitivity/tolerance of the species). However, they are not applied in estuarine and marine environments so often as it happens, for instance, with the macrofauna indices based on indicator species (e.g. AMBI, Borja et al., 2000; BENTHIX, Simboura and Zenetos, 2002; BQI, Rosenberg et al., 2004), mainly because they tend to be highly site and situation specific (e.g. NemaSPEAR, Höss et al., 2011). Nematode indicator genera are those that take advantage of the stressed situation at a particular site to dominate in numbers at the expense of other nematode genera, being normally referred as opportunistic (Gyedu-Ababio and Baird, 2006). Although some generalizations can be done regarding tolerance of some nematode genera, indicator species need to be identified or confirmed by laboratory experiments (Gyedu-Ababio and Baird, 2006), since the use of such indicators requires caution because, more often than not, species being examined may occur naturally in relatively high densities in estuaries (as stated for macrobenthic communities by Marques et al., 2009). As no reliable methodology to know at which level the existence of those indicator species can be well represented in a community that is not really affected by any kind of pollution exists, a degree of subjectivity is implicit (Warwick, 1993). Nevertheless, despite the difficulty in ascribing indicator genera to specific disturbance events, Höss et al. (2011) developed a metric (NemaSPEAR) to assess pollution in freshwater soft sediments. Based on the proportion of nematode species at risk (i.e., only occurring in samples with low toxic stress and rarely in polluted samples) in a field-based approach, relating nematodes with metal and organic contamination (translated into ecotoxicological units), the NemaSPEAR development was supported by the SPEAR classification of macroinvertebrates, which considers ecological and

ecotoxicological information (sensitivity to toxicants, generation time and migration ability) (Liess and Von der Ohe, 2005). Later, Losi (2013) developed a similar index in order to assess the effects of contamination on marine sediments and to evaluate their ecological quality. According to these authors, this stressor-specific metric provides a tool for assessing the cause-and-effect relationship between the chemical status or toxic stress of a certain site and its ecological status (Höss et al., 2011; Losi, 2013). Nevertheless, further research aiming to select a suite of nematode genera sensitive to chemical contamination to be used in monitoring programs is desirable (Losi, 2013).

Although the described indices have proven relevant and present their advantages and utility, they are focused on single impact factors thus, reflecting only single aspects of the community under observation. On the other hand, a multimetric approach would give an integrated analysis of the biological community of a site (Karr and Chu, 1999). Its ability to integrate different biological descriptors (e.g. taxa richness, diversity measures, proportion of sensitive and tolerant species, trophic structure) where each single component metric is predictably and reasonably related to specific impacts caused by environmental alterations (Hering et al., 2006), makes the multimetric index a more reliable tool than assessment methods based on single metrics.

In fact, a multimetric approach would offer detection capability over a wide range of stressors and a more complete picture of the ecosystem (Vlek et al., 2004), because it can, potentially, reflect multiple effects of human impact on different aspects of the structure and function of ecosystems (Barbour et al., 1995; 1999; Klemm et al., 2003). The final multimetric index could encompass several metrics which are known to reflect the system's ecological status. By their integration in a unique index, several aspects of the system could be analyzed and, according to the main objective of its application and knowledge of the system, different weights to the metrics could be applied, allowing for a holistic interpretation of the system.

4. Suggestions for future research

This thesis represents a step further towards the knowledge about meiobenthic communities, particularly free-living nematodes. But, as often is the case, it also highlighted new paths that could be followed in order to further improve knowledge on meiobenthic communities, enhancing its application in diverse assessment studies.

1) Improvement of taxonomic identification processes

Taxonomic impediment constitutes a serious handicap in the evaluation of biodiversity (Rodman and Cody, 2003; Wheeler et al., 2004) and of free-living marine nematodes (Coomans, 2000; 2002). The use of tools such as the NeMys online identification key (Steyaert et al., 2005) allowed scientists to benefit from a bulk of identification keys, schemes, pictures and texts regarding several nematode species/genera.

However, special attention is now being directed towards genetic and molecular investigations. Nevertheless, the traditional morphological identification cannot be set aside, but instead be complemented by these approaches. Furthermore, if a suite of genera would to be identified as the focus of monitoring in ecological assessments studies, these identification techniques could be of extreme importance for future ecological assessment studies, by reducing costs and time of the analyses and increasing identification accurateness. According to Neher et al. (2004), the identification of sentinel nematode genera would be imperative as they would be classified accordingly their tolerance or sensitivity to different types of disturbance, leading to a reduction of the number of genera that need to be enumerated and identified.

2) The ecological role of nematodes in the ecosystem and in food webs

The extent to which several factors affect the distribution of nematode communities demands further investigation, namely in understanding how

communities under different degrees of disturbance change in response to shifts in natural conditions. Following identification improvement, physiological and ecological information of particular species should also be obtained. In this sense, microcosms experiments are a comprehensive step for testing, in controlled conditions, a hypothesis originated from field patterns (Daehler and Strong, 1996), and allow complex interactions to be disentangled.

Special care is however necessary when extrapolating the results because different processes occur and have different impacts at different scales. Consequently, larger field approaches, like mesocosms, should also be done, to validate the extrapolation of small-scale studies to larger ones, and to allow large scale modelling of the effects of different parameters.

The role and quantitative importance of free-living nematodes in marine and estuarine soft sediments remain enigmatic due to lack of empirical evidence on the feeding habits and trophic position of most nematode species (Moens et al., 2005). Morphological and behavioural observations (e.g. Jensen, 1987; Moens and Vincx, 1997) have been leading to changes in the trophic guilds described by Wieser (1953), which clearly acknowledges the need for an accurate classification of resources utilization and trophic level of nematodes. Therefore, studies evaluating nematode trophic positions in estuarine foodwebs and resource utilization should be encouraged, making use of stable isotope, using the natural abundance of stable carbon and nitrogen isotopes, and fatty acid composition.

3) Promoting well designed studies: the importance of fine temporal scale and long-term time studies.

Due to the short nematode life cycle nematodes are the ideal biological group to survey when fast responses are needed (for example, the impact of acute pollution sources). Nevertheless, long–term studies are essential to understand the complex processes that operate in dynamic systems such as estuaries. Long-term studies allow studying the impacts of natural events (e.g. climatic events like floods and droughts) on the communities, understanding if, and how, they affect the structure and distribution patterns of nematode communities. Moreover, they

would also allow to monitor the communities in different phases (pre, during and post disturbance), exploring the dynamic response of free-living nematode communities to the disturbance events. Such studies can also be useful to test different management and restoration techniques to understand the best way to circumvent negative impacts of stressors.

> "The wider public will turn their attention to the meiobenthos when we understand that we must present meiobenthology not just as a fascinating scientific field, but also as an extremely useful one for solving important problems."
>
> Giere, 2009

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Annexes

Annex 1. Pair-wise tests results for each of the two- factors Permanova tests ("area" with 5 levels, and "sampling occasion" with 6 levels, as fixed factors) for all variables analyzed. A. Meiofauna and B.Nematodes.

A. Meiofauna

Composition	
Factor "Area"	
Oligohaline	Su06≠Au06, Sp07, Su09, Au09; Au06≠Wi07
Mesohaline	Su06≠Au06, Wi07, Sp07, Su09, Au09; Au06≠Su09; Wi07≠Au09
Polyhaline NA	Su06≠Au06, Sp07
Polyhaline SA	Wi07≠ Au06, Sp07, Su09, Au09
Euhaline	All pairs were different, except Wi07-Su09, Sp07-Su09 and Su09-Au09
Factor "Sampling occasion"	
Summer 2006	Oligo≠Meso, Poly NA, Eu; Eu≠Meso, Poly SA
Autumn 2006	Oligo≠Meso, Poly NA, Poly Sa; Poly NA≠Eu
Winter 2007	Poly SA≠Oligo, Meso, Poly NA, Eu;
Spring 2007	Oligo≠Poly NA, Poly Sa, Eu
Summer 2009	Oligo≠Meso, Poly NA, Poly SA, Eu; Meso≠Poly NA, Poly SA, Eu
Autumn 2009	Oligo≠Meso, Poly NA, Poly SA, Eu; Meso≠Poly NA, Poly SA; Eu≠Poly SA

B. Nematodes

Total Density	
Factor "Area"	
Oligohaline	Su06>Au06, Sp07, Su09, Au09; Wi07>Au06,Au09
Mesohaline	Au09<Su09, Au06
Polyhaline NA	No differences
Polyhaline SA	Wi07>Au06, Sp07, Su09, Au09
Euhaline	Su06>Au06, Sp07, Su09; Au06<Wi07, Sp07, Su09, Au09; Wi07>Au09
Factor "Sampling occasion"	
Summer 2006	Oligo<Meso, Poly NA, Eu; Meso<Eu
Autumn 2006	Oligo<Meso, Poly NA, Poly SA, Eu
Winter 2007	Oligo<Eu; Poly SA>Oligo, Meso, Poly NA, Eu
Spring 2007	Oligo<Poly NA, Poly SA, Eu
Summer 2009	Oligo<Meso, Poly NA, Poly SA, Eu; Meso<Poly NA, Poly SA
Autumn 2009	Oligo<Meso, Poly NA, Poly SA, Eu; Meso<Poly NA, Poly SA
Number of genera	
Factor "Area"	
Oligohaline	Au06<Su06, Wi07, Au09; Wi07>Su09
Mesohaline	Wi07>Su09, Au09
Polyhaline NA	Su09<Su06, Wi07
Polyhaline SA	Wi07<Au09
Euhaline	Su06>Sp07
Factor "Sampling occasion"	
Summer 2006	Eu>Oligo, Meso
Autumn 2006	No differences
Winter 2007	Poly SA<Oligo, Meso, Poly NA, Eu

Spring 2007	No differences
Summer 2009	Eu>Oligo, Meso, Poly NA, Poly SA
Autumn 2009	No differences
Trophic structure	
Factor "Area"	
Oligohaline	Sp07≠ Su06, Au06, Wi07, Su09, Au09; Au09≠Wi07, Su09
Mesohaline	Su06≠Au06, Wi07, Sp07; Au06≠Su09, Au09; Wi07≠Su09, Au09; Sp07≠Su09
Polyhaline NA	Su06≠Su09, Au09; Au06≠So07, Au09; Su09≠Au09
Polyhaline SA	Sp07≠Au06, Au09
Euhaline	Su06≠Su09, Au09; Au06≠Su09, Au09; Wi07≠Su09
Factor "Sampling occasion"	
Summer 2006	Oligo≠Meso; Meso≠Poly NA, Eu; Poly NA≠Eu
Autumn 2006	Meso≠Poly NA, Poly SA, Eu
Winter 2007	Poly NA≠Oligo, Eu
Spring 2007	Poly NA≠Meso, Eu; Poly SA≠Meso
Summer 2009	Oligo≠Meso, Poly NA, Poly SA, Eu
Autumn 2009	Oligo≠Meso, Poly NA, Poly SA; Poly NA≠Poly SA, Eu
Composition	
Factor "Area"	
Oligohaline	All pair were different, except Wi07-Sp07
Mesohaline	Su06≠Au06, Wi07, Au09; Au06≠Sp07, Su09, Au09; Au09 ≠Wi07, Sp07
Polyhaline NA	Au06≠Sp07, Su09, Au09; Wi07≠Sp07, Su09, Au09; Su09≠Sp07, Au09
Polyhaline SA	All pairs were different, except Su09-Au09
Euhaline	Su06≠Au06, Wi07, Sp07, Su09, Au09; Wi07≠Su09, Au09
Factor "Sampling occasion"	
Summer 2006	All pairs were different
Autumn 2006	All pairs were different, except Poly NA-Poly SA and Eu-Poly SA
Winter 2007	Oligo≠Eu, Poly SA; Meso≠Poly SA, Eu; Poly NA ≠ Eu, Poly SA; Eu≠Poly SA
Spring 2007	All pairs except Eu-Poly SA
Summer 2009	All pairs were different
Autumn 2009	All pairs were different, except Poly NA – Poly SA
Margalef Index (d)	
Factor "Area"	
Oligohaline	Su06<Wi07, Au09; Au06<Wi07, Au09; Su09<Wi07, Au09
Mesohaline	No differences
Polyhaline NA	Wi07>Su09
Polyhaline SA	Wi07<Sp07, Su09, Au09; Su09<Sp07, Au09
Euhaline	no differences
Factor "Sampling occasion"	
Summer 2006	No differences
Autumn 2006	Oligo>Poly NA, Poly SA; Eu>Poly SA
Winter 2007	Oligo >Poly NA, Poly SA, Eu; Eu> Poly SA
Spring 2007	No differences
Summer 2009	Oligo> Poly NA, Poly SA; Meso>Poly NA, Poly SA; Eu> Poly NA, Poly SA
Autumn 2009	Oligo> Meso, Poly NA, Poly SA

Shannon-Wiener Index (H')	
Factor "Area"	
Oligohaline	(all pairs are different except Meso-Poly SA)
Mesohaline	
Polyhaline NA	
Polyhaline SA	
Euhaline	
Factor "Sampling occasion"	
Summer 2006	-
Autumn 2006	-
Winter 2007	-
Spring 2007	-
Summer 2009	-
Autumn 2009	-
Index of Trophic Diversity (ITD)	
Factor "Area"	
Oligohaline	Independently of the sampling occasion:
Mesohaline	Meso>Eu; Poly NA >Eu; Oligo >Eu; Poly NA >Poly SA
Polyhaline NA	
Polyhaline SA	
Euhaline	
Factor "Sampling occasion"	
Summer 2006	-
Autumn 2006	-
Winter 2007	-
Spring 2007	-
Summer 2009	-
Autumn 2009	-
Maturity Index (MI)	
Factor "Area"	
Oligohaline	Su09>Sp07, Au09
Mesohaline	Su06<Au06, Wi07; Au06>Sp07, Su09, Au09; Wi07>Su09, Au09
Polyhaline NA	Au06>Sp07, Su09, Au09
Polyhaline SA	No differences
Euhaline	Au09<Wi07, Sp07
Factor "Sampling occasion"	
Summer 2006	Meso<Oligo, Eu
Autumn 2006	No differences
Winter 2007	Poly NA<Meso, Eu
Spring 2007	Eu>Meso, Poly NA, Poly SA; Poly NA< Poly SA
Summer 2009	Oligo>Meso, Poly NA, Poly SA, Eu; Poly NA<Poly SA
Autumn 2009	Oligo>Meso, Poly NA; Poly NA<Poly SA

Annex 2. Environmental variables measured at each sampling station and sampling occasion in the Mondego estuary

Sampling occasions labels: [August 2006 (Au06), November 2006 (Nv06), March 2007 (Mr07), June 2007 (Ju07), September 2009 (Sp09) and December 2009 (Dc09)].

Areas labels: [Euhaline (E), Polyhaline South Arm (P SA), Polyhaline North Arm (P NA), Mesohaline (M) and Oligohaline (O)].

Env. Variables labels: Sal, salinity; DO, dissolved oxygen; NH_4^+, ammonia; NO3-, nitrate; NO2-, nitrite; PO43-, phosphate; Si, silicates; Chl *a*, cholorophyll *a*; OM, organic matter; S+C, silt+clay; FS, fine sand; MS, medium sand; CS, coarse sand; G, gravel.

Sampling occasion	Area	Station	Sal	DO	NH_4^+	NO_3^-	NO_2^-	PO_4^{3-}	Si	Chl *a*	OM	S+C	FS	MS	CS	G
Au06	**E**	**4**	32.2	8.7	0.04	0.21	0	0.03		3.98	0.9	1.99	60.94	27.6	7.9	1.57
Au06	**P NA**	**13**	31.8	8.8	0.04	0.04	0	0.03	0	3.46	1.4	4.53	17.54	21.98	26.29	29.66
Au06	**M**	**18**	18.5	7.3	0.08	0.37	0.01	0.05	0	5.94	4.8	12.18	59.07	16.2	11.41	1.14
Au06	**M**	**19**	15.2	7.5	0.08	0.42	0.01	0.05	0	7.33	3.8	10.38	74.12	14.36	0.91	0.22
Au06	**O**	**21**	5.5	6.3	0.1	0.71	0.02	0.06	0	10.72	3	5.13	38.95	15.91	1.65	38.35
Au06	**O**	**23**	0.1	6.2	0.13	1.37	0.05	0.09	0	21.56	4.1	6.74	64.42	16.91	3.09	8.84
Au06	**O**	**25**	0	6.5	0.19	1.33	0.06	0.09	0	33.13	0.2	0.17	1.88	16.22	45.99	35.75
Nv06	**E**	**4**	29.3	8.37	0.01	0	0	0.01	0.55	3.06	0.51	0.09	8.9	30.61	47.31	13.1
Nv06	**P SA**	**6**	20	8.27	0.03	0.46	0.01	0.03	1.48	3.46	1.51	3.66	36.34	24.21	28.06	7.73
Nv06	**P SA**	**7**	12.1	7.7	0.08	0.91	0.02	0.05	1.48	2.86	0.23	0.15	14.15	39.76	37.31	8.63
Nv06	**P SA**	**9**	10.2	8.05	0.26	0.45	0.03	0.06	1.51	5.61	5.87	4.07	66.35	29.14	0.43	0.01
Nv06	**P NA**	**12**	31.2		0	0.03	0	0.01	0.42	4.43	0.25	0.02	3.67	62.83	26.13	7.35
Nv06	**P NA**	**13**	29.2		0	0.29	0	0.02	0.61	4.05	0.91	0.2	3.88	68.19	25.79	1.94
Nv06	**M**	**18**	0		0.11	1.6	0.02	0.03	4.21	8.74	0.39	0.03	0.37	7.92	61.61	30.07
Nv06	**M**	**19**	0		0.06	1.3	0.01	0.03	2.28	8.43	0.03	0.01	0.32	6.98	58.28	34.41
Nv06	**O**	**21**	0		0.04	1.2	0.01	0.03	2.32	7.84	1.67	2.33	9.23	28.64	41.29	18.51
Nv06	**O**	**23**	0		0.04	1.1	0.01	0.03	2.97	8.85	0.29	0.01	0.16	15.08	81.56	3.19
Nv06	**O**	**25**	0.1		0.04	1.58	0.01	0.04	3.03	7.31	0.51	0.02	0.62	9.63	79	10.73
Mr07	**E**	**4**	33.6	10.9	0.01	0.25	0.01	0.02	0.43	4.81	2.39	57.3	25.4	0.3	15.6	1.4
Mr07	**P SA**	**6**	20.1	21	0.08	0.78	0.02	0.04	1.8	7.21	3.03	60.8	19.1	0.4	18.6	1.2
Mr07	**P SA**	**9**	19.5	9.7	0.17	0.51	0.03	0.05	1.57	11.49	4.03	41	35.9	1	19.8	2.3
Mr07	**P NA**	**12**	34.3	13.3	0	0.2	0.01	0.03	0.36	11.14	0.27	1.3	26.6	1.2	60.3	10.7
Mr07	**M**	**18**	0.5	11	0.07	1.76	0.02	0.04	3.67	16.19	0.25	0	0.4	12.7	61.7	25.2
Mr07	**O**	**21**	0	11.1	0.13	1.93	0.02	0.04	3.78	15.05	0.38	0.8	21	0.8	69.2	8.2
Mr07	**O**	**25**	0	9.5	0.06	1.91	0.02	0.03	1.71	10.82	0.32	0.1	3.2	16.4	47.7	32.6

Ju07	**E**	**4**	32.8	9.7	0	0.09	0	0	0.26	5.77	0.81	0.76	14.33	44.71	36.99	3.22
Ju07	**P SA**	**6**	30.3	8.3	0.02	0.11	0	0.01	0.49	6.89	1.19	5.39	34.26	22.25	27.98	10.13
Ju07	**P SA**	**7**	26.8	7.9	0.07	0.24	0.01	0.03	0.81	9.64	4.76	19.17	50.78	14.78	12.08	3.19
Ju07	**P SA**	**9**	25.2	6.6	0.15	0.12	0.03	0.04	1.2	9.61	7.28	19.34	63.56	13.99	2.01	1.1
Ju07	**P NA**	**12**	32.6	10	0	0.02	0	0.01	0.21	4.20	0.41	0	8.98	78.8	9.1	3.12
Ju07	**P NA**	**13**	32.3	9.9	0	0.04	0	0.01	0.29		0.27	0.05	2.97	46.35	48.99	1.64
Ju07	**M**	**18**	23.8	8	0.03	0.53	0.01	0.03	0.96	5.95	0.38	0.26	0.73	7.28	42.35	49.38
Ju07	**O**	**21**	3.7	6.2	0.05	1.49	0.01	0.07	2.66	7.49	0.34	0.36	2.3	21.6	62.62	13.12
Ju07	**O**	**25**	0.4	6	0.07	1.54	0.02	0.07	2.87	10.20	0.26	0	1.16	10.29	44.99	43.57

Sp09	**E**	**4**	35.2	8.6	0.03	0.09	0.01	0.01	0.24	3.06	1.2	4.1	44.5	33.1	16.4	2
Sp09	**P SA**	**6**	33.3	7.7	0.03	0.15	0.02	0.01	0.31	3.39	7.41	11.8	55	25.3	7	0.9
Sp09	**P SA**	**7**	29.8	7.5	0.07	0.21	0.02	0.03	0.69	5.90	3.2	14	38.8	13	29.7	4.5
Sp09	**P SA**	**9**	31.5	7.3	0.21	0.18	0.02	0.04	1.07	5.75	5.01	8.6	49.1	14.8	24.1	3.4
Sp09	**P NA**	**12**	35.48	5.75	0.01	0.14	0.01	0	0.15	2.63	3.59	13.1	39	15	23.8	9.2
Sp09	**P NA**	**13**	37.56	5.91	0.01	0.25	0.02	0.01	0.22	3.83	0.66	1.9	23.1	29.3	34.4	11.2
Sp09	**M**	**18**	27.54	5.56	0.06	0.37	0.01	0.04	0.68	5.32	5.95	14.7	50.8	10.3	17.4	6.8
Sp09	**M**	**19**	19.27	5.19	0.11	0.72	0.01	0.05	1.28	15.13	4.4	12.6	53.5	12.1	17.3	4.5
Sp09	**O**	**21**	2.97	5.57	0.16	1.09	0.02	0.06	0.94	11.64	0.57	6.6	64.5	16	8.9	4.1
Sp09	**O**	**23**	0.84	5.95	0.11	1.16	0.02	0.06	1.73	16.70	5.38	10.6	49.1	22.4	16.8	1.1
Sp09	**O**	**25**	0.18	6.7	0.11	1.21	0.04	0.06	0.78	16.53	3	0.7	0.6	3.9	48.4	46.4

Dc09	**E**	**4**		9.1	0.02	0.58	0.02	0.03	0.63	3.03	3.76	5.31	44.58	33.47	14.93	1.71
Dc09	**P SA**	**6**		9	0.03	0.61	0.02	0.05	1.52	1.20	8.48	9.31	52.49	30.25	6.99	0.95
Dc09	**P SA**	**7**		9.6	0.1	0.83	0.03	0.06	2.11	4.50	2.78	18.22	46.57	10.19	22.87	2.15
Dc09	**P SA**	**9**		8.5	0.28	0.82	0.06	0.08	2.47	5.16	4.64	17.94	59.92	12.02	10.02	0.1
Dc09	**P NA**	**12**	28.1	6.58	0	0.33	0	0.02	0.8	2.52	5.5	22.94	47.81	9.1	17.55	2.6
Dc09	**P NA**	**13**	28.2	6.35	0.01	0.71	0	0.02	0.84	2.12	3.52	12.78	28.63	19.02	29.03	10.54
Dc09	**M**	**18**	0	8.61	0.09	1.46	0.01	0.04	3.18	15.86	5.86	12.28	60.61	10.55	15.37	1.19
Dc09	**M**	**19**	0	7.54	0.08	1.48	0.01	0.04	2.92	5.21	8.11	10.5	61.1	9.91	17.4	1.09
Dc09	**O**	**21**	0	8.19	0.26	1.54	0.01	0.05	3.39	3.59	4.83	8.42	52.43	18.41	13.66	7.08
Dc09	**O**	**23**	0	8.59	0.27	1.52	0.01	0.05	2.93	2.55	2.15	8.09	59.73	17.5	14.14	0.54
Dc09	**O**	**25**	0	7.5	0.18	1.77	0.01	0.03	3.06	1.97	1.53	6.64	30.18	43.4	17.27	2.52

Annex 3. Results from the two-way PERMANOVA tests, considering A. the taxonomic levels, B. each functional group, and C) the combined biological traits matrix. Values in bold were significant at $p<0.05$.

	Source of variation	Degrees of freedom	Sum of squares	Mean squares	Pseudo-F	P (perm)
A.						
Genus	Area	4	99146	24787	16.713	0.0001
	Sampling occasion	5	37523	7504.5	5.0602	0.0001
	Area x sampling occasion	19	60822	3201.2	2.1585	**0.0001**
	Residual	139	20614	1483		
	Total	167	41815			
Family	Area	4	96501	24125	20.295	0.0001
	Sampling occasion	5	29277	5855.3	4.9258	0.0001
	Area x sampling occasion	19	48590	2557.4	2.1514	**0.0001**
	Residual	139	165230	1188.7		
	Total	167	353170			
Order	Area	4	69339	17335	28.024	0.0001
	Sampling occasion	5	12784	2556.7	4.1334	0.0001
	Area x sampling occasion	19	25806	1358.2	2.1958	**0.0001**
	Residual	139	85980	618.56		
	Total	167	198160			
B.						
Feeding type	Area	4	42924	10731	16.048	0.0001
	Sampling occasion	5	14864	2972.9	4.4458	0.0001
	Area x sampling occasion	19	20676	1088.2	1.6274	**0.0018**
	Residual	139	92948	668.69		
	Total	167	172400			
Life strategy	Area	4	58697	14674	25.11	0.0001
	Sampling occasion	5	12252	2450.4	4.193	0.0001
	Area x sampling occasion	19	23309	1226.8	2.0992	**0.0002**
	Residual	139	81232	584.4		
	Total	167	178500			
Tail shape	Area	4	56001	14000	21.195	0.0001
	Sampling occasion	5	13697	2739.4	4.1472	0.0001
	Area x sampling occasion	19	21570	1135.2	1.7186	**0.0015**
	Residual	139	91816	660.54		
	Total	167	185800			
Body shape	Area	4	43288	10822	19.964	0.0001
	Sampling occasion	5	11376	2275.2	4.1972	0.0001
	Area x sampling occasion	19	18927	996.18	1.8377	**0.0022**
	Residual	139	75348	542.07		
	Total	167	150540			
C.						
Multi- trait	Area	4	49010	12252	19.787	0.0001
	Sampling occasion	5	12889	2577.8	4.1629	0.0001
	Area x sampling occasion	19	21239	1117.8	1.8052	**0.0005**
	Residual	139	86072	619.22		
	Total	167	171230			

Annex 4. Pair-wise tests results for each of the two-way Permanova tests ("area" with 5 levels and "sampling occasion" with 6 levels, as fixed factors) considering A. the taxonomic levels, B. each functional group, and C. the combined biological traits matrix.

Areas: Oligohaline (O), Mesohaline (M), Polyhaline NA (PNA), Polyhaline SA (PSA) and Euhaline (E).
Sampling occasions: August 2006 (Au06), November 2006 (Nv06), March 2007 (Mr07), June 2007 (Ju07), September 2009 (Sp09) and December 2009 (Dc09).

A. Taxonomic levels	
Genus	
Factor "Area"	
Oligohaline	Au06≠Nv06, Mr07, Ju07, Sp09, Dc09; Nv06≠Ju07, Sp09, Dc09; Mr07≠Sp09, Dc09; Ju07≠Sp09, Dc09
Mesohaline	Au06≠Nv06, Mr07, Ju07, Dc09; Nv06≠Ju07, Sp09, Dc09; Mr07≠Sp09, Dc09; Ju07≠Dc09
Polyhaline NA	Au06≠Ju07, Sp09, Dc09; Mr07≠Ju07, Sp09, Dc09; Ju07≠Sp09; Sp09≠Dc09
Polyhaline SA	Au06≠Mr07, Ju07, Sp09, Dc09; Mr07≠Ju07, Sp09, Dc09; Ju07≠Sp09, Dc09
Euhaline	no differences
Factor "Sampling occasion"	
August 2006	O≠M, PNA, E; M≠PNA, E
November 2006	O≠M, PNA, PSA, E; M≠PNA, PSA, E; NA≠E
March 2007	O≠M, PNA, PSA, E; PSA≠M, PNA, E
June 2007	O≠PNA, PSA, E; M≠PNA, PSA; PNA≠PSA, E
September 2009	all different
December 2009	all different except PNA=PSA
Family	
Factor "Area"	
Oligohaline	all≠except Mr07=Ju07
Mesohaline	Au06≠Nv06, Mr07, Dc09; Nv06≠Sp09, Dc09; Mr07≠Sp09, Dc09; Ju07≠Dc09
Polyhaline NA	Nv06≠Ju07, Sp09, Dc09; Mr07≠Ju07, Sp09, Dc09; Ju07≠Sp09
Polyhaline SA	Nv06≠Mr07, Ju07, Sp09; Mr07≠Ju07, Sp09, Dc09; Ju07≠Sp09
Euhaline	no differences
Factor "Sampling occasion"	
August 2006	all ≠ except PNA=E
November 2006	O≠M, PNA, PSA, E; M≠PNA, PSA, E;PNA≠E
March 2007	O≠PNA, PSA, E; PSA≠M, PNA, E
June 2007	O≠PNA, PSA, E; M≠PNA, PSA; PNA≠PSA, E; PSA≠E
September 2009	all ≠
December 2009	all ≠ except PNA=PSA
Order	
Factor "Area"	
Oligohaline	Au06≠Nv06, Ju07, Dc09; Mr07≠Sp09; Ju07≠Sp09, Dc09; Sp09≠Dc09
Mesohaline	Au06≠Nv06, Mr07,Dc09; Nv06≠Dc09; Mr07≠Sp09, Dc09
Polyhaline NA	no differences
Polyhaline SA	Mr07≠Nv06, Ju07, Sp09, Dc09
Euhaline	no differences

Factor "Sampling occasion"	
August 2006	O≠M, PNA, E; M≠E
November 2006	O≠M, PNA, PSA, E; PNA≠E
March 2007	O≠PSA, E; PSA≠M, PNA, E
June 2007	O≠PNA, PSA, E
September 2009	O≠M, PNA, PSA, E; M≠PNA, PSA
December 2009	O≠M, PNA, PSA, E; M≠PNA, PSA

B. Functional group	
Feeding type	
Factor "Area"	
Oligohaline	Au06≠all seasons
Mesohaline	Au06≠Mr07, Dc09; Dc09≠Nv06, Mr07
Polyhaline NA	no differences
Polyhaline SA	Mr07≠Au06, Ju07, Sp09, Dc09
Euhaline	Au06≠Nv06, Mr07, Ju07, Sp09, Dc09; Nv06≠Mr07, Ju07, Sp09, Dc09; Mr07≠Ju07, Sp09, Dc09
Factor "Sampling occasion"	
August 2006	O≠M; E≠O, M, PNA
November 2006	O≠M,PNA, PSA; E≠PNA
March 2007	PSA≠O, M, PNA; PNA≠E
June 2007	O≠PNA, PSA, E
September 2009	O≠M, PNA, PSA, E; M≠PNA, PSA
December 2009	O≠PNA, PSA, E; ≠PNA, PSA
Life strategy	
Factor "Area"	
Oligohaline	Au06≠Nv06, Ju07, Sp09, Dc09; Nv06≠Ju07; Sp09≠Ju07, Dc09
Mesohaline	Au06≠Mr07,Sp09, Dc09; Nv06≠Sp09, Dc09; Mr07≠Sp09, Dc09
Polyhaline NA	no differences
Polyhaline SA	Mr07≠Nv06, Ju07, Sp09, Dc09
Euhaline	Au06≠Nv06, Mr07, Ju07, Sp09, Dc09; Mr07≠Nv06, Ju07, Dc09
Factor "Sampling occasion"	
August 2006	O≠M, PNA, E; E≠ M, PNA
November 2006	O≠M,PNA, PSA; PNA≠E
March 2007	O≠PSA, M≠PNA, PSA; PNA≠PSA, E
June 2007	O≠PNA, PSA, E; PNA≠E
September 2009	O≠M, PNA, PSA, E; M≠PNA, PSA
December 2009	O≠PNA, PSA, E; M≠PNA, PSA
Tail shape	
Factor "Area"	
Oligohaline	Au06≠Nv06, Ju07, Sp09, Dc09; Sp09≠Nv06, Ju07, Dc09
Mesohaline	Dc09≠Au06, Nv06, Mr07
Polyhaline NA	no differences
Polyhaline SA	Mr07≠Nv06, Ju07, Sp09, Dc09
Euhaline	Au06≠Nv06, Mr07, Ju07,Sp09, Dc09; Mr07≠Nv06, Ju07, Dc09; Ju07≠Dc09
Factor "Sampling occasion"	
August 2006	O≠M, PNA, E; E≠ M, PNA
November 2006	O≠M, PNA, PSA; PNA≠E
March 2007	O≠PSA, E; PSA≠M, PNA
June 2007	O≠PNA, PSA, E
September 2009	O≠M, PNA, PSA, E; M≠PNA, PSA
December 2009	O≠M, PNA, PSA, E; ≠PSA, E

Body shape	
Factor "Area"	
Oligohaline	Au06≠Nv06, Ju07, Sp09, Dc09; Sp09≠Ju07, Dc09
Mesohaline	Dc09≠Au06, Nv06, Mr07
Polyhaline NA	no differences
Polyhaline SA	Mr07≠Au06, Ju07, Sp09, Dc09
Euhaline	Au06≠Nv06, Mr07, Ju07,Sp09, Dc09; Nv06≠Mr07, Ju07, Sp09; Mr07≠Ju07, Dc09
Factor "Sampling occasion"	
August 2006	O≠M, PNA, E; E≠ M, PNA
November 2006	O≠M, PNA, PSA; PNA≠E
March 2007	PSA≠O, M, PNA, E; E≠PNA
June 2007	O≠PNA, PSA, E
September 2009	O≠M, PNA, PSA, E; M≠PNA, PSA
December 2009	O≠PNA, PSA, E; M≠PSA

C. Multi-trait	
BTA	
Factor "Area"	
Oligohaline	Au06≠Nv06, Ju07, Sp09, Dc09; Sp09≠Ju07, Dc09
Mesohaline	Dc09≠Au06, Nv06, Mr07
Polyhaline NA	no differences
Polyhaline SA	Mr07≠Au06, Ju07, Sp09, Dc09
Euhaline	Au06≠Nv06, Mr07, Ju07, Sp09, Dc09; Nv06≠Mr07, Ju07, Sp09; Mr07≠Ju07, Dc09
Factor "Sampling occasion"	
August 2006	all different except M=PNA
November 2006	O≠M, PNA, PSA; PNA≠E
March 2007	PSA≠O, M, PNA, E; E≠PNA
June 2007	O≠PNA, PSA, E
September 2009	O≠M, PNA, PSA, E; M≠PNA, PSA
December 2009	O≠PNA, PSA, E; M≠PSA

Annex 5. Genera determined by SIMPER analysis as contributing the most to the similarity within Areas. Shaded boxes: percent similarity (bold) and the genera that contributed to the similarity in each group. Non-shaded box, percent dissimilarity (bold) between areas and the genera that contributed to the total dissimilarity (cut-off percentage: 75%).

	Euhaline	Polyhaline South Arm	Polyhaline North Arm	Mesohaline	Oligohaline
Euhaline	**45.79%** *Daptonema* *Sabatieria* *Viscosia* *Sphaerolaimus* *Linhomoeus* *Oncholaimellus* *Dichromadora* *Anoplostoma* *Terschellingia* *Molgolaimus*				
Polyhaline South Arm	**54.50%** *Sabatieria* *Metachromadora* *Terschellingia* *Daptonema* *Sphaerolaimus* *Anoplostoma* *Ptycholaimellus* *Oncholaimellus* *Linhomoeus* *Molgolaimus* *Microlaimus* *Viscosia* *Axonolaimus* *Dichromadora* *Prochromadorella* *Odontophora* *Paracyatholaimus* *Paracanthonchus* *Calyptronema* *Aegialoalaimus*	**55.70%** *Sabatieria* *Sphaerolaimus* *Daptonema* *Viscosia* *Anoplostoma* *Terschellingia*			

Polyhaline North Arm	**55.09%**	**44.57%**	**58.15%**	
	Sabatieria	*Sabatieria*	*Sabatieria*	
	Metachromadora	*Daptonema*	*Daptonema*	
	Daptonema	*Terschellingia*	*Dichromadora*	
	Anoplostoma	*Sphaerolaimus*	*Sphaerolaimus*	
	Sphaerolaimus	*Dichromadora*	*Terschellingia*	
	Dichromadora	*Ptycholaimellus*		
	Oncholaimellus	*Anoplostoma*		
	Viscosia	*Viscosia*		
	Molgolaimus	*Metachromadora*		
	Ptycholaimellus	*Linhomoeus*		
	Terschellingia	*Leptolaimus*		
	Microlaimus			
	Linhomoeus			
	Leptolaimus			
	Axonolaimus			
	Prochromadorella			
	Odontophora			
	Paracanthonchus			
	Aegialoalaimus			
	Chromadora			
Mesohaline	**62.39%**	**59.72%**	**59.06%**	**48.18%**
	Sabatieria	*Sabatieria*	*Sabatieria*	*Daptonema*
	Anoplostoma	*Sphaerolaimus*	*Daptonema*	*Anoplostoma*
	Metachromadora	*Terschellingia*	*Anoplostoma*	*Dichromadora*
	Daptonema	*Daptonema*	*Sphaerolaimus*	*Terschellingia*
	Terschellingia	*Anoplostoma*	*Dichromadora*	*Viscosia*
	Ptycholaimellus	*Ptycholaimellus*	*Terschellingia*	*Paracyatholaimus*
	Sphaerolaimus	*Paracyatholaimus*	*Ptycholaimellus*	
	Viscosia	*Dichromadora*	*Paracyatholaimus*	
	Linhomoeus	*Linhomoeus*	*Viscosia*	
	Molgolaimus	*Viscosia*	*Leptolaimus*	
	Dichromadora	*Metachromadora*	*Metachromadora*	
	Oncholaimellus	*Axonolaimus*	*Spilophorella*	
	Paracyatholaimus	*Leptolaimus*		
	Microlaimus			
	Axonolaimus			
	Prochromadorella			
	Odontophora			
	Paracanthonchus			
	Aegialoalaimus			
	Mesodorylaimus			
	Aponema			
	Leptolaimus			

Oligohaline	**75.40%**	**75.82%**	**74.79%**	**66.17%**	**36.60%**
	Sabatieria	*Sabatieria*	*Sabatieria*	*Daptonema*	*Daptonema*
	Daptonema	*Sphaerolaimus*	*Sphaerolaimus*	*Anoplostoma*	*Mesodorylaimus*
	Metachromadora	*Terschellingia*	*Dichromadora*	*Dichromadora*	*Ptycholaimellus*
	Viscosia	*Daptonema*	*Daptonema*	*Terschellingia*	*Anoplostoma*
	Sphaerolaimus	*Mesodorylaimus*	*Mesodorylaimus*	*Mesodorylaimus*	*Sabatieria*
	Mesodorylaimus	*Viscosia*	*Viscosia*	*Paracyatholaimus*	*Dichromadora*
	Linhomoeus	*Anoplostoma*	*Terschellingia*	*Ptycholaimellus*	*Paracyatholaimus*
	Oncholaimellus	*Ptycholaimellus*	*Anoplostoma*	*Sphaerolaimus*	*Viscosia*
	Molgolaimus	*Linhomoeus*	*Leptolaimus*	*Viscosia*	*Neotobrilus*
	Anoplostoma	*Dichromadora*	*Metachromadora*	*Axonolaimus*	
	Terschellingia	*Metachromadora*	*Ptycholaimellus*	*Leptolaimus*	
	Microlaimus	*Paracyatholaimus*	*Paracyatholaimus*	*Sabatieria*	
	Ptycholaimellus	*Neotobrilus*	*Linhomoeus*	*Neotobrilus*	
	Dichromadora	*Mononchus*	*Neotobrilus*	*Spilophorella*	
	Axonolaimus	*Halalaimus*	*Axonolaimus*	*Mononchus*	
	Paracyatholaimus			*Chromadorita*	
	Odontophora			*Laimydorus*	
	Paracanthonchus			*Chromadorina*	
	Prochromadorella			*Plectus*	
	Halalaimus			*Ascolaimus*	
	Aegialoalaimus				
	Aponema				
	Leptolaimus				
	Chromadora				
	Calyptronema				

Annex 6. Nematode genera determined by two-way SIMPER analysis as contributing the most to the similarity/dissimilarity of nematode communities within (A) sampling occasions and (B) areas. Shaded boxes: percent similarity (bold) and the genera that contributed to the similarity in each group. Non-shaded box: percent dissimilarity (bold) and the genera that contributed to the total dissimilarity (cut-off percentage: 70%).

A.

	September 2009		**December 2009**		**March 2010**	
September 2009	**66.96%**					
	Sabatieria	23.91				
	Daptonema	14.46				
	Sphaerolaimus	13.12				
	Paracomesoma	6.67				
	Terschellingia	6.64				
	Paralinhomoeus	6.42				
December 2009	**47.43%**		**60.18%**			
	Ptycholaimellus	6.86	*Sabatieria*	15.51		
	Sabatieria	6.40	*Daptonema*	12.46		
	Daptonema	6.37	*Sphaerolaimus*	11.11		
	Metachromadora	6.04	*Ptycholaimellus*	9.09		
	Viscosia	5.60	*Viscosia*	8.78		
	Chromadora	4.93	*Dichromadora*	6.28		
	Terschellingia	4.89	*Metachromadora*	5.42		
	Sphaerolaimus	4.63	*Paralinhomoeus*	5.16		
	Paralinhomoeus	4.40				
	Dichromadora	3.84				
	Paracomesoma	3.57				
	Microlaimus	3.40				
	Anoplostoma	3.40				
	Axonolaimus	2.95				
	Desmolaimus	2.92				
March 2010	**49.46%**		**57.02%**		**63.95%**	
	Sabatieria	16.64	*Sabatieria*	10.97	*Daptonema*	24.93
	Terschellingia	9.10	*Ptycholaimellus*	7.45	*Sphaerolaimus*	13.52
	Daptonema	7.91	*Sphaerolaimus*	6.59	*Sabatieria*	13.48
	Paracomesoma	6.77	*Daptonema*	5.58	*Viscosia*	11.48
	Sphaerolaimus	6.76	*Paralinhomoeus*	5.49	*Dichromadora*	11.32
	Paralinhomoeus	5.82	*Viscosia*	5.19		
	Dichromadora	4.87	*Metachromadora*	5.17		
	Ptycholaimellus	4.67	*Chromadora*	4.62		
	Viscosia	4.29	*Terschellingia*	3.98		
	Linhomoeus	3.89	*Dichromadora*	3.64		
			Anoplostoma	3.56		
			Microlaimus	3.16		
			Axonolaimus	2.88		
			Desmolaimus	2.69		

B.

	Zostera		Intermedia		Armazens		Montante	
Zostera	**63.88%**							
	Sabatieria	20.11						
	Daptonema	19.02						
	Sphaerolaimus	13.78						
	Dichromadora	10.45						
	Viscosia	6.21						
	Paralinhomoeus	6.15						
Intermédia	**44.82%**		**62.33%**					
	Sabatieria	9.81	*Daptonema*	15.30				
	Ptycholaimellus	8.41	*Sabatieria*	10.98				
	Daptonema	6.61	*Dichromadora*	10.82				
	Terschellingia	6.54	*Sphaerolaimus*	10.00				
	Dichromadora	5.38	*Ptycholaimellus*	8.97				
	Sphaerolaimus	4.77	*Viscosia*	8.50				
	Viscosia	4.71	*Paracomesoma*	5.19				
	Axonolaimus	4.55	*Paralinhomoeus*	4.18				
	Paralinhomoeus	4.40						
	Anoplostoma	4.09						
	Calyptronema	3.86						
	Linhomoeus	3.63						
	Chromadora	3.33						
Armazéns	**47.70%**		**46.30%**		**61.14%**			
	Viscosia	7.23	*Daptonema*	7.69	*Sabatieria*	16.55		
	Sabatieria	7.05	*Sabatieria*	5.95	*Daptonema*	15.41		
	Daptonema	6.98	*Viscosia*	5.75	*Viscosia*	13.07		
	Anoplostoma	6.55	*Dichromadora*	5.47	*Sphaerolaimus*	11.10		
	Ptycholaimellus	6.13	*Ptycholaimellus*	4.98	*Anoplostoma*	9.41		
	Terschellingia	5.91	*Paralinhomoeus*	4.43	*Dichromadora*	5.75		
	Dichromadora	5.70	*Calyptronema*	4.31				
	Paralinhomoeus	4.25	*Anoplostoma*	4.20				
	Sphaerolaimus	4.23	*Axonolaimus*	4.18				
	Nemanema	3.41	*Paracomesoma*	4.14				
	Linhomoeus	3.33	*Nemanema*	3.74				
	Metalinhomoeus	3.13	*Sphaerolaimus*	3.63				
	Metachromadora	2.90	*Linhomoeus*	3.52				
	Axonolaimus	2.64	*Terschellingia*	2.93				
	Paracomesoma	2.60	*Oncholaimellus*	2.82				
			Odontophora	2.70				
Montante	**42.13%**		**46.33%**		**45.59%**		**66.77%**	
	Ptycholaimellus	11.99	*Sabatieria*	11.81	*Sabatieria*	8.68	*Sabatieria*	21.98
	Sabatieria	8.58	*Daptonema*	7.97	*Daptonema*	8.32	*Daptonema*	20.13
	Daptonema	8.31	*Dichromadora*	6.52	*Ptycholaimellus*	7.91	*Sphaerolaimus*	15.39
	Dichromadora	7.80	*Sphaerolaimus*	6.48	*Sphaerolaimus*	6.88	*Ptycholaimellus*	10.72
	Terschellingia	7.08	*Ptycholaimellus*	6.28	*Metachromadora*	6.07	*Viscosia*	8.31
	Sphaerolaimus	6.08	*Metachromadora*	5.72	*Viscosia*	5.22		
	Metachromadora	5.37	*Viscosia*	4.06	*Anoplostoma*	4.95		
	Viscosia	4.90	*Terschellingia*	4.01	*Terschellingia*	4.62		
	Anoplostoma	4.63	*Paracomesoma*	4.00	*Paralinhomoeus*	4.29		
	Paralinhomoeus	4.07	*Axonolaimus*	3.95	*Nemanema*	3.92		
	Linhomoeus	3.36	*Paralinhomoeus*	3.84	*Dichromadora*	3.49		
			Calyptronema	3.65	*Metalinhomoeus*	2.64		
			Linhomoeus	3.40	*Linhomoeus*	2.46		
					Axonolaimus	2.26		